

**TRANSPOSON INSERTION AT THE PROMOTER OF A MYB  
TRANSCRIPTION FACTOR RESULTS IN ECTOPIC ANTHOCYANINS  
ACCUMULATION IN PURPLE CAULIFLOWER (*Brassica oleracea* L var.  
*botrytis*)**

**A Dissertation**

**Presented to the Faculty of the Graduate School  
of Cornell University**

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Doctor of Philosophy**

**by**

**Li Wei Chiu**

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**Li Wei Chiu, Ph. D.**

**Cornell University 2010**

Flavonoids such as anthocyanins possess significant health benefits to humans and play important physiological roles in plants. An interesting *purple* (*pr*) gene mutation in cauliflower confers an abnormal pattern of anthocyanin accumulation, giving rise to intense purple color in very young tissues, curds, and seeds. Through a combination of candidate analysis and fine-mapping, we have isolated the *pr* gene and verified it via functional complementation in wild type *Arabidopsis* and cauliflower. The *pr* gene was found to encode a R2R3-MYB transcription factor that exhibited tissue-specific expression, consistent with anthocyanin accumulation pattern in the mutant. Comparison of the DNA sequences between the WT and mutant alleles revealed that the mutation was caused by an autonomous DNA transposon insertion in the promoter region of the *pr* allele. The sequence rearrangement generated several *cis*-acting R response elements (RRE), which may provide new binding sites for regulatory proteins in conferring enhanced expression of the *pr* gene. GUS activity assay demonstrated that the promoter activity was correlated with the numbers of RREs in the promoter region. The upregulation of the *pr* gene activated the expression of a bHLH transcription factor (*BoTT8*), which in turn activated a subset of anthocyanin structural genes *BoF3'H*, *BoDFR*, and *BoLDOX* to produce the striking mutant

phenotype. Yeast two-hybrid assays showed that the PURPLE (PR) protein directly interacted with BoTT8 and exhibited stronger binding activity than other BoMYBs isolated. The successful isolation of the *pr* gene has provided important insights for understanding the regulation of anthocyanin accumulation for breeding *Brassica* vegetables with enhanced health-promoting properties and visual appeal. Our discovery also has also demonstrated the involvement of transposable elements in gene regulation for phenotypic change in plants.



## **BIOGRAPHICAL SKETCH**

Li-Wei Chiu was born in 1982 in Taipei, Taiwan. She lived close to a library when she was little and thus became an early and avid reader. Her interest in biology burgeoned during childhood when she started reading science fiction, and the concept of cloning caught her imagination. In junior high school, the film “Jurassic Park” and the subsequent reading of the relevant subject matter illustrated to her that such genetic transformations are not merely dreams of novelists; thereafter, she developed a keen interest in biology and decided to major in biology at Tunghai University, Taiwan. Her internship in 2002 at the Botany Section of the National Museum of Natural Science in Taiwan reaffirmed her interest in plant biology, leading to her decision to pursue higher education to become a plant biologist in the future. She grew up in Taiwan where diversity, nutrition and quality play important roles in the food culture and national economy. Her childhood dream, her interests in plant, and her cultural influence led her to join Dr. Li Li’s laboratory at Cornell University as a Master student in 2004 with focus on the research of vegetable nutrition improvement through molecular biology techniques. To fulfill her goal of becoming an independent plant biologist, she transferred to the Ph.D. program in 2007 and continued working on the same project. She is now looking forward to receiving her postdoctoral training at Pioneer Hi-Bred Company to achieve her research goal, which is to provide healthier and better quality foods to the world.

Dedicated to all my teachers, family members and friends, for their support and faith  
in me.

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## LIST OF ABBREVIATION

PAL	Phenylalanine ammonia lyase
C4H	Cinnamate 4-hydroxylase
4CL	4-coumarate CoA ligase
CHS	Chalcone synthase
CHI	Chalcone isomerases
F3H	Flavonone 3-hydroxylase
F3'H	Flavonoid 3'-hydroxylase
F3'5'H	Flavonoid 3',5'-hydroxylase
DFR	Dihydroflavonol reductase
LDOX	Anthocyanidin synthase
UGT	UDP glucose-flavonol glucosyl transferase
ACT	Acyltransferase
OMT	O-malonyltransferase
FLS	Flavonol synthase
TTG1	Transparent TESTA GLABRA 1
TTG2	Transparent TESTA GLABRA 2
TT8	Transparent TESTA 8
TT2	Transparent TESTA 2
PAP1	Production of Anthocyanin Pigment 1
PAP2	Production of Anthocyanin Pigment 2
GL3	Glabra 3
EGL3	Enhancer of Glabra 3
RNS1	Ribonuclease 1
FUS3	Fusca 3

ANL2	Anthocyaninless 2
bHLH	Basic helix-loop-helix
bZIP	Basic Leucine Zipper
RRE	R response elements
WBM	WD40-bHLH-MYB
RFLP	Restriction fragment length polymorphism
SNP	Single nucleotide polymorphism
ARMS-PCR	Amplification refractory mutation system - polymerase chain reaction

# **CHAPTER 1**

## **ANTHOCYANINS BIOSYNTHESIS IN PLANTS**

### **1.1. INTRODUCTION**

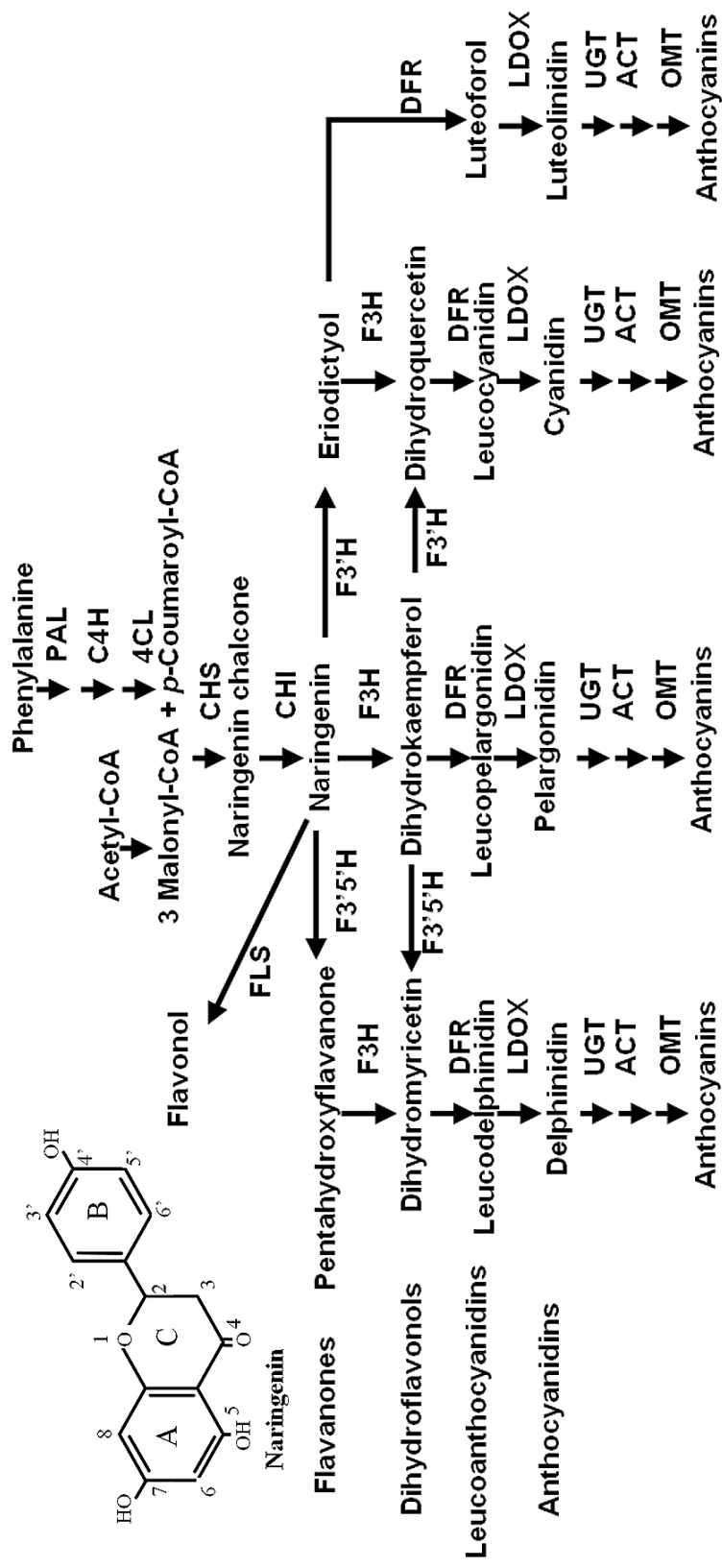
Anthocyanins are the largest group of water-soluble pigments in the plant kingdom and are responsible for the blue, red, and purple color of many fruits, vegetables, grains, flowers, and leaves. They play an important role in protecting the photosynthetic apparatus from damage by stress, improving the foliar nutrient re-absorption during senescence, and acting as attractants to pollinators and for seed dispersal (Harborne and Williams 2000, Steyn et al. 2002, Steyn 2009, Winkel-Shirley 2001). In recent years, numerous studies have shown that anthocyanins have numerous health benefits to humans, such as improvement of night vision and near-sightedness (Lee et al. 2005, Matsumoto et al. 2003), protection against neurological disorders (Cho et al. 2003, Kang et al. 2006), prevention of cardiovascular disease (Lazze et al. 2006, Youdim et al. 2000b), and reduction in the risk of cancer (Cooke et al. 2005, Fimognari et al. 2008, Hou 2003, Thomasset et al. 2009). According to recent data (Centers for Disease Control and Prevention, 2006), the top two leading causes of death in America are heart diseases (26%) and cancer (23.1%). In this case, functional foods containing health-beneficial phytochemicals such as anthocyanins have become of great interest to the society (Kong et al. 2003).

Anthocyanins are the most prominent class of flavonoids, and the distinguishing features for different anthocyanins include the hydroxyl groups, the attached sugars, the position of attachment, and the attached aliphatic or aromatic acids to the sugars. A total of 19 types of anthocyanins have been found in nature; however, only six of them are commonly found in higher plants, namely delphinidin, pelargonidin, cyanidin, peonidin, malvidins and petunidin (Tanaka et al. 2008).

Cyanidin, delphinidin and pelargonidin are present in 80% of pigmented leaves, 69% of fruits, and 50% of flowers (Williams and Grayer 2004). The most common anthocyanidins in edible parts of plants is cyanidin, present in 50% of the cases (Kong et al. 2003).

## **1.2. ANTHOCYANIN BIOSYNTHETIC PATHWAY GENES**

The anthocyanin biosynthetic pathway (Figure 1.1) shares a number of common biosynthetic steps with various flavonoid subpathways, including the formation of naringenin chalcone by chalcone synthase (CHS) and the isomerizations of naringenin chalcone into naringenin by chalcone isomerase (CHI), followed by hydroxylation and reduction by flavanone 3-hydroxylase (F3H), flavonoids 3',5'-hydroxylase (F3',5'H), flavonoids 3'-hydroxylase (F3'H), and dihydroflavonol 4-reductase (DFR) to yield leucoanthocyanidin, the immediate precursor for anthocyanin biosynthesis. The subsequent conversions into colored anthocyanins are catalyzed by leucoanthocyanidin dioxygenase (LDOX), UDP-glucosyltransferase (UGT), acyltransferase (ACT), and O-methyltransferase (OMT), resulting in the formation of anthocyanins with increased stability and water solubility (Ben-Meir et al. 2002, Springob et al. 2003, Tanaka et al. 2008). The anthocyanin biosynthetic pathway enzymes appear to work together as a complex. Phenylalanine ammonia-lyase (PAL), cinnamate 4-hydroxylase (C4H), CHS, and 3-GT have been found with weak membrane associations (Ro et al. 2001). They are suggested to be loosely associated with the endoplasmic reticulum by P450 proteins. Yeast two-hybrid experiments have revealed the existence of protein interactions between CHS, CHI, DFR and F3H in *Arabidopsis thaliana* (Burbulis and Winkel-Shirley 1999).



**Figure 1.1.** Anthocyanin biosynthetic pathway. PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; F3'5'H, flavonoid 3',5'-hydroxylase; DFR, dihydroflavonol 4-reductase; LDOX, leucoanthocyanidin dioxygenase; FLS, flavonol synthase; UGT, UDP-glucosyltransferase; ACT, acyltransferase; OMT, O-malonyltransferase



### ***1.2.1. Chalcone synthase (CHS)***

Chalcone synthase is the enzyme that catalyzes the first step of the flavonoid and anthocyanin biosynthesis pathway. It catalyzes the sequential condensation of one molecule of coumaroyl-CoA and three molecules of malonyl-CoA to produce naringenin chalcone (2',4,4',6'-tetrahydroxychalcone) (Springob et al. 2003). CHS is a homodimeric protein with two 40-45 kDa functionally independent subunits and belongs to the polyketide synthases family (PKS) (Tropf et al. 1995). The PKS family proteins play an important role in plants. This family proteins include stilbene synthases (Schoppner and Kindl 1984), pyrone synthases (Eckermann et al. 1998), acridone synthases (Junghanns et al. 1995), benzalacetone synthases (Abe et al. 2001), and valerophenone synthases (Paniego et al. 1999). Because of the high sequence similarity among the PKS family proteins, it is impossible to predict the function based on the sequence alone. Also, in the database, many proteins that have been classified as CHS-like proteins do not all share the same function (Springob et al. 2003).

The CHS dimer consists of two functional parts, the coumaroyl-binding pocket and cyclization pocket. There are three residues, C164, H303 and N336, in both pockets and they account for the decarboxylation and condensation function (Eckermann et al. 2003). C164 provides the binding between the phenylpropanoid starter and the polyketide chain while C164 and H303 form an ion pair that helps to stabilize the negatively charged thiolate, which is required for the acyltransferase activity. H303 and N336 also interact together to provide the driving force behind the decarboxylation of malonyl-CoA and to stabilize the enolate of acetyl-CoA carbanion within the reaction.

### **1.2.2. Chalcone isomerase (CHI)**

Chalcone isomerase catalyzes the cyclization of naringenin chalcone to (2S)-naringenin (4',5,7-trihydroxyflavanone). It is a 24 to 29 kDa monomer enzyme with no homology to other proteins and has only been found in higher plants (Springob et al. 2003, Tanner 2004). There are two major kinds of CHI, one in non-legumes plants and the other in legume plants. Both are capable of converting naringenin chalcone to (2S)-naringenin, but only the legume plant CHI can also convert isoliquiritigenin to (2S)-5-deoxyflavanone (Dixon et al. 1988). In *Arabidopsis thaliana*, mutation of CHI will result in a phenotype lacking flavonoid (Dong et al. 2001). However, in *Zea mays*, even without detectable CHI activity, anthocyanin synthesis nevertheless occurs (Grotewold et al. 1998).

### **1.2.3. Flavanone 3-hydroxylase (F3H)**

Flavanone 3-hydroxylase is a soluble 2-oxoglutarate-dependent dioxygenase with a molecular weight of approximately 40.9 kDa and requires the presence of  $\text{Fe}^{2+}$ ,  $\text{O}_2$ , 2-oxoglutarate and ascorbate to function properly (Lukacin et al. 2000). It catalyzes the hydroxylation of the flavanones' ([2S]-naringenin or [2S]-eriodictyol) position 3, and then converts it to dihydroflavonols ([2R, 3R]-dihydrokaempferol or [2R, 3R]-dihydroquercetin) (Springob et al. 2003, Tanner 2004).

### **1.2.4. Flavone 3'-hydroxylase (F3'H) and flavonoid 3', 5'-hydroxylase (F3'5'H)**

F3'H and F3'5'H are P450-dependent monooxygenases that require NADPH and  $\text{O}_2$  for the hydroxylation activity of the B-ring of many of the flavonoids (Springob et al. 2003, Tanner 2004). F3'H is about 56.8 kDa and initiates the pathway for cyanidin-type anthocyanin synthesis; it catalyzes the 3'-hydroxylation of flavanone, dihydroflavonol, flavone and flavonol (Tanner 2004). F3'5'H leads the synthesis of

delphinidin-type anthocyanins and it catalyzes the 3' and 5' position hydroxylation of flavanone and dihydroflavonol (Stotz and Forkmann 1982).

#### ***1.2.5. Dihydroflavonol 4-reductase (DFR)***

Dihydroflavonol 4-reductase produces leucoanthocyanidins by reducing dihydroflavonols in the presence of NADPH. DFR belongs to a single-domain reductase/ epimerase/ dehydrogenase protein family (Fischer et al. 1988), and exhibits strong specificity for different dihydroflavonols between species. For example, some DFRs in plant species such as *Petunia*, *Nicotiana*, and *Cymbidium* are incapable of reducing dihydrokaempferol to pelargonidin (Heller and Forkmann 1993). Recent evidence has shown that a change of a single amino acid will enable the use of dihydrokaempferol as substrate by this type of DFR in *Gerbera* (Johnson et al. 2001).

#### ***1.2.6. Leucoanthocyanidin dioxygenase (LDOX)***

Leucoanthocyanidin dioxygenase is also known as anthocyanidin synthase (ANS) and belongs to the 2-oxoglutarate-dependent oxygenases family of proteins. It is a soluble nonheme iron enzyme of about 40.5 kDa. It catalyzes the oxidation of leucoanthocyanidin to produce anthocyanidins. ANS is not only responsible for anthocyanidin biosynthesis, but also for the proanthocyanidin biosynthetic pathway (Springob et al. 2003, Tanner 2004). The study of the ANS mechanism of oxidation was not successfully completed until 1999, when ANS isolated from *Perilla frutescens* was overexpressed in *E. coli*. It catalyzes the oxidation in the presence of ferrous iron, 2-oxoglutarate, ascorbate, and acidification with HCl (Saito et al. 1999). Another study showed that ANS also exhibited hydroxylation activity at the leucoanthocyanidin C-3 position (Nakajima et al. 2001). Under *in vitro* conditions,

ANS can not only catalyze the conversion of leucoanthocyanidin to anthocyanidin but also produce dihydroflavonol and flavonol (Turnbull et al. 2000).

#### ***1.2.7. Glycosyltransferases (GT)***

Anthocyanin pigments are usually present in glycosylated forms *in vivo* at the 3-O-position or 5-O-position (Springob et al. 2003, Tanner 2004). The glycosylation of the 3-O-position is necessary for further modifications such as acylation and methylation. It is performed by UDP-glucose anthocyanidin 3-O-glucosyltransferase, and can increase the stability, and influence the color and antioxidant activity of the pigments (Saito and Yamazaki 2002). The glycosylation of the 5-O-position requires the formation of anthocyanidin 3-Glu as a substrate. It is catalyzed by the anthocyanin 5-O-glucosyltransferase to produce the complex for co-pigmentation (Springob et al. 2003).

#### ***1.2.8. Acyltransferase (ACT)***

Acyltransferase is an acyl-CoA-dependent enzyme which is responsible for the acylation of anthocyanins (Nakayama et al. 2003). Two types of acyl substituent of anthocyanins exist: the aromatic and aliphatic acyl groups. Acylation of the anthocyanins can help to increase the intra- and/or inter-molecular stacking (co-pigmentation), the water solubility, uptake into vacuoles, bioavailability, and protection against degradation by glycosidase. Co-pigmentation of different molecules such as flavones and flavonols results in an intense blue color (Saito and Yamazaki 2002).

#### ***1.2.9. O-Methyltransferase (OMT)***

O-methyltransferase mediates the methylation of anthocyanins, which results in a slight reddening effect and reduces the chemical reactivity of the phenolic hydroxyl groups. It can also increase the stability and water solubility of anthocyanins for vacuole storage (Lam et al. 2007). Two classes of methyltransferases have been found (Joshi and Chiang 1998, Lam et al. 2007): type 1 OMTs are homodimers (about 38 to 43 kDa), and do not require divalent cations for activity (NDong et al. 2003), whereas type 2 OMTs have a lower molecular mass (23 to 27 kDa), and are cation-dependent (Ye et al. 1994).

### **1.3. ANTHOCYANIN REGULATORY GENES**

The anthocyanin biosynthetic pathway has been shown to be regulated by many transcription factors. Most of those transcription factors are not only responsible for the anthocyanin synthetic pathway but also other traits. The R2R3-MYB and basic helix-loop-helix (bHLH) proteins are the most well known among those transcription factors (Allan et al. 2008, Broun 2005, Koes et al. 2005, Mol et al. 1998). Results from yeast two hybrid assays have suggested a model where the R2R3-MYB protein, bHLH protein, and WD40 protein work together as a transcription complex, WBM, to regulate anthocyanin biosynthetic genes (Baudry et al. 2006, Zimmermann et al. 2004). In maize, the R2R3-MYB family proteins, Colorless 1 (C1)/ Purple leaf (Pl), and bHLH family proteins, RED (R)/ Booster (B), work together to induce the expression of the anthocyanin biosynthetic pathway genes (Chandler et al. 1989, Cone et al. 1993, Ludwig and Wessler 1990). JAF13 and AN2 found in *Petunia hybrida* induce the late anthocyanin biosynthetic pathway genes but not the early genes, compared with maize R and C1 (Quattrocchio et al. 1999). The bHLH family proteins are essential for the C1/Pl function (Cone et al. 1993); however, other R2R3-MYB homologue P can

induce the expression of the anthocyanin structural genes independently (Grotewold et al. 1994). In dicots, MYB305 and MYB340 function like maize P; they can activate the early genes of the flavonoid biosynthetic pathway without the bHLH partners (Moyano et al. 1996).

### ***1.3.1. MYB family proteins***

Several kinds of MYB family proteins are involved in the regulation of anthocyanin biosynthesis. An *Arabidopsis* subgroup 6 R2R3-MYB protein, PAP1, which has a sequence similar to that of *Petunia* AN2 (Quattrocchio et al. 1999), can activate the phenylpropanoid pathway, and therefore resulting in purple pigmentation throughout the whole plant. A study using a chimeric PAP1 repressor has shown that PAP1 regulates many structure genes in the anthocyanin synthetic pathway, such as *CHS*, *DFR* and *LDOX* in *Arabidopsis* siliques (Borevitz et al. 2000). It can also regulate the expression of some proanthocyanidin synthesis related genes such as *BAN* and *TT2* (Nesi et al. 2000). The MYB and bHLH proteins, required for the proanthocyanidin accumulation in the seed by regulating the expression of *DFR* and *BAN*, are encoded individually by the *TT2* and *TT8* loci (Baudry et al. 2004, Nesi et al. 2000). Over-expression of *TT2* in *Arabidopsis* results in over-expression of *TT8*, but the reverse does not hold true (Nesi et al. 2001). A total of four subgroup 6 R2R3-MYB proteins have been found in *Arabidopsis*, including PAP1, PAP2, MYB113 and MYB114 (Stracke et al. 2001), all activators of anthocyanin biosynthetic pathway (Borevitz et al. 2000, Dubos et al. 2008, Gonzalez et al. 2008, Hemm et al. 2001, Matsui et al. 2008). Recently, several orthologs have been found in different species, such as *MdMYB10* in apple (Chagne et al. 2007, Espley et al. 2007), *VlMYBA1* in grape (Azuma et al. 2008, Kobayashi et al. 2004), *IbMYB2* in sweet potato (Mano et al. 2007), and *AmROSEA1* in snapdragon (Schwinn et al. 2006). The expression of *MdMYB10* in apple can be

regulated by itself as well as by bHLH proteins (Espley et al. 2009), suggesting that the regulatory network is complex.

Only a few anthocyanin biosynthesis repressors have been found. FaMYB1 (Aharoni et al. 2001), a MYB family protein found in strawberry, has high sequence homology to *Arabidopsis thaliana* subgroup 4 R2R3-MYB protein, AtMYB4, and *Antrirrhinum majus*, AmMYB308, which act as transcriptional inhibitors. Over-expression of FaMYB1 in tobacco reduces the expression of the late anthocyanin biosynthetic genes. FaMYB1 has been shown to interact with petunia JAF13 and AN1 based on findings from the yeast two-hybrid assay. AtMYB4 and AmMYB308 contain the LNL[E/D]L motif in their C-terminal domain, suggesting their involvement in repression of expression (Hemm et al. 2001, Tamagnone et al. 1998). Recently a R3-MYB protein MYBL2 in *Arabidopsis* has been identified as a repressor as well (Dubos et al. 2008, Matsui et al. 2008). The mechanism by which MYBL2 down regulates anthocyanin biosynthesis is through competition for bHLH protein binding with the subgroup 6 R2R3-MYB proteins in the WBM complex.

### **1.3.2. bHLH proteins**

The subgroup IIIb bHLH proteins in *Arabidopsis* include TT8, EGL3 and GL3 (Heim et al. 2003), each of which has multiple functions controlling different traits. TT8 controls seed pigmentation and seed coat mucilage; EGL3 controls seed coat mucilage, initiation of trichomes, and differentiation of bald epidermal cell files; and GL3 controls initiation of trichomes and differentiation of bald epidermal cell files (Zhang et al. 2003). Moreover, they are all positive regulators of the anthocyanin biosynthetic pathway. Several orthologs have been found in different species as well, e.g. IpbHLH1 and IpbHLH2 in morning glory plants (Park et al. 2007) with different petal and seed coat colors. *IpbHLH1* is genetically closer to *AtTT8* and petunia *AN1* while *IpbHLH2*

is closer to *AtEGL3*, *AtGL3*, and petunia *JAF13*. However, they seem to function differently since the mutation of petunia *ANI* is deficient in acidification of the vacuoles (Spelt et al. 2002), but vacuolar alkalization in the epidermal flower of *IpbHLH2* mutants appears to occur normally (Park et al. 2007), suggesting species specific regulation. The expression of bHLH genes is auto-regulated or regulated by other bHLH proteins, together with MYB transcription factors (Baudry et al. 2006). The autoregulation of regulatory proteins may turn out to be a common mechanism for regulation (Baudry et al. 2006, Espley et al. 2009).

### **1.3.3. WD40 proteins**

WD40 proteins, such as AN11 from petunia and TTG1 from *Arabidopsis*, also play an important role in regulation of the anthocyanin biosynthesis. *AN11* has been hypothesized to be an upstream gene of *AN2* (de Vetten et al. 1997) while TTG1, in contrast, modifies the activity of bHLH protein such as TT8 (Walker et al. 1999). One particular model suggests that the MYB, bHLH, and WD40 proteins form a complex to regulate the structural genes of anthocyanin synthesis (Lepiniec et al. 2006, Winkel-Shirley 2001). However, different combinations of various R2R3-MYB and bHLH proteins may result in different functions such as anthocyanin or proanthocyanidin synthesis, seed coat mucilage, differentiation of bald root epidermal cell files, and initiation of trichomes (Zhang et al. 2003). It is still not very clear how these transcription factors work together and the interaction seem complex.

## **1.4. TRANSPORT AND ACCUMULATION OF ANTHOCYANINS**

Most anthocyanin synthesis related enzymes are located in the cytosol or in the endoplasmic reticulum membrane. The anthocyanins synthesized in the cytosol must be transported to the vacuoles for storage (Springob et al. 2003, Tanaka et al. 2008,



Tanner 2004). However, the mechanism of anthocyanin transport and accumulation in vacuoles is still poorly understood. *Zea mays* BZ2 (Marrs et al. 1995) and *Petunia hybrida* An9 (Mueller et al. 2000) connect cyanidin-3-*O*-glucoside with glutathione; therefore the product can be transported across the tonoplast by a Mg-ATP-requiring glutathione pump. *TT19* in *Arabidopsis thaliana*, which has 50% homology with An9, encodes a Phi glutathione S-transferase (Kitamura et al. 2004). After the anthocyanins are transferred into the vacuoles, they bind with a protein matrix known as anthocyanic vacuolar inclusions (AVIs) (Markham et al. 2000, Pourcel et al. 2009), which may exhibit selectivity for acylated anthocyanins. VP24, a sweet potato 24 kDa protein found in AVI-containing cells, has been hypothesized to be involved in the transport or stable accumulation of anthocyanins in vacuoles (Nozue et al. 2003). In carrot, a pH-dependent transporter involved in the anthocyanin transport into vacuolar has also been found (Hopp and Seitz 1987).

The pH of the vacuole influences the color of the anthocyanin pigment. The vacuolar lumen is usually more acidic than its surrounding cytoplasm (Tanaka et al. 2008). Mutation of a pH regulatory protein in petunia results in blue instead of red flower under acidic vacuolar lumen conditions (Quattrocchio et al. 2006). In contrast, the *Ipomea* blue flower color is regulated by the PURPLE (PR) protein, which is a putative  $\text{Na}^+/\text{H}^+$  pump that transports sodium ion into and protons out of the vacuole (Yoshida et al. 2005). The cell shape and anthocyanins stored in the vacuole also influence the flower color. Snapdragon flower color can differ based on different cell type (conical or flat cells), or the nature of the light reflection (Gorton and Vogelmann 1996). The color differences in the lisianthus petal are due to the different distributions of AVIs; the reduction or absence of AVIs results in a lighter purple color compared to the normal blackish-purple flower color (Zhang et al. 2006). Besides, the induced

flower color of the Rhapsody in Blue rose cultivar by ageing is due to the progressive accumulation of anthocyanins in the AVIs (Gonnet 2003).

## **1.5. ENVIRONMENTAL FACTORS CONTROL THE ACCUMULATION OF ANTHOCYANINS IN PLANTS**

As a large group of plant pigments, anthocyanins play an important role in pollination and seed dispersal by attraction to animals. However, anthocyanins not only exist in the flower and fruits of plants, but also appear in the vegetative tissues. The functions of anthocyanins in the vegetative tissue are still unclear. Anthocyanins are accumulated under high light, low temperature, nutrient deficiency, and wounding and pathogen attack conditions (Close and Beadle 2003, Steyn et al. 2002). The function of anthocyanins in the vegetative tissues has been hypothesized based on their responses to these conditions.

### ***1.5.1. High light***

High light condition induces anthocyanin synthesis in vegetative tissues, and the anthocyanin level varies with different light exposure on the plants or on a single leaf. Anthocyanin, PSI and PSII synthesis are regulated by their specific signal transduction pathways through phytochrome-mediated activation (Bowler et al. 1994). During the early seedlings growth stages of seedlings which is the stage where the seedlings are most susceptible to light-induced stress, anthocyanins are synthesized, and thus suppress greening (Drummherrel and Mohr 1985). The suppression of anthocyanin synthesis starts after chlorophyll accumulation, and the emphasis shifts to carbon assimilation. The signaling pathway for anthocyanin synthesis is less sensitive the signaling compounds than other pathways (Bowler et al. 1994). Therefore, a stronger signal, i.e. high light, is required for the activation of the anthocyanin synthesis.

The distribution of anthocyanins in plant tissues is dependent on the tissue specific expression of regulatory genes, which respond to environmental and developmental factors (Mol et al. 1996). Anthocyanin synthesis is also a cell-autonomous process which allows for a localized accumulation (Nick et al. 1993). Anthocyanins are generally accumulated in the leaf upper epidermis, the tissue that is directly exposed to the light (Chalker-Scott 1999). However, accumulation can also occur in the mesophyll cells (McClure 1975) and vascular parenchyma of the leaf midrib (Baudry et al. 2004).

Photoinhibition means that over-excitation of the photosynthetic apparatus, which is caused by the energy capture rate being greater than the electron transport and dissipation, manifests itself as a repression of photosynthesis (Long et al. 1994). Photoinhibitory conditions may result in the formation of reactive oxygen species and therefore cause damage to the cells (Foyer et al. 1994). Anthocyanins can modify the quality and quantity of light that is incident on the chloroplasts by absorbing the green, ultraviolet, and some of the blue light (McClure 1975). They seem unable to absorb red light. Therefore, it has been suggested that anthocyanins act as photoprotective pigments through their ability to absorb visible light. By exposing pods of red and green *Bauhinia variegata* under red, blue-green or white light, a study showed that red pods exhibit higher tolerance to intensities of blue-green and white light than green pods (Smillie and Hetherington 1999). However, there was no significant difference under red light treatment. In senescing leaves, anthocyanins can also reduce photo-oxidative damage and therefore allow plants to improve the efficiency of nutrient retrieval from the leaves (Hoch et al. 2001).

Although ultraviolet light can be absorbed by anthocyanins, it is unlikely that anthocyanins are the major contributors in protecting plants from ultraviolet light damage. Increased UV-B radiation might result in anthocyanin level reduction; in

contrast, the accumulation of colorless phenols and flavonoids can increase the absorbance of UV-B (Moorthy and Kathiresan 1997).

#### ***1.5.2. Low temperature***

Low temperature is yet another factor that causes anthocyanin accumulation. Low temperature ( $<10^{\circ}\text{C}$ ) enhances the transcription of the anthocyanin structural genes, but post-transcriptional regulation requires a higher temperature ( $25^{\circ}\text{C}$ ) (Christie et al. 1994). The temperature required varies among different species (Steyn et al. 2002). Low temperatures can result in decreasing activity of light capture and  $\text{O}_2$  evolution enzymes; therefore, under the same light condition, photoinhibition increases (Huner et al. 1998). The increasing level of anthocyanins provides photoprotection against photoinhibition in plants.

#### ***1.5.3. Nutrient deficiency***

Phosphorus and nitrogen deficiency can result in anthocyanin accumulation. In an *Arabidopsis* mutant which is unable to maintain an adequate internal P level, a 100-fold increase in anthocyanin accumulation was observed compared to the normal phenotype (Zakhleniuk et al. 2001). P and nitrogen deficiency can lead to growth reduction, carbohydrate accumulation, sugar-repression of photosynthesis, decreasing levels of Calvin Cycle enzymes, and increased susceptibility to photostress (Close and Beadle 2003, Steyn et al. 2002). N deficiency affects the expression of anthocyanin synthesis related genes while P deficiency affects energy metabolism caused by the lack of P for involving in high energy compounds (Sughiarto et al. 1990). With increased anthocyanin accumulation, maize exhibits higher P deficiency tolerance (Gaume et al. 2001).

#### ***1.5.4. Wounding and pathogen attack***

Anthocyanin accumulation can be found in wounding or pathogen attacked tissues. However, it does not appear to be responsible for pathogen or disease resistance. For example, there is no survival difference among aphids which were fed with artificial diets with high concentration of anthocyanins compared with normal diets (Costa-Arbulu et al. 2001). However, Coley and Aide found that the amount of leaf-cutting ant significantly decreases with increased levels of anthocyanins in the leaves of the plant species in their habitat (Costa-Arbulu et al. 2001). This result suggests that the anthocyanins provide a visual signal for the levels of other compounds, such as high phenols, low chlorophyll and low N, to the herbivores.

### **1.6. ANTHOCYANINS BENEFITS TO HUMAN HEALTH**

Numerous studies have shown that anthocyanins pose health benefits to humans, such as protection of DNA by preventing radical hydroxyl attack, improvement of night vision and near-sightedness, protection against neurological disorders, prevention of cardiovascular disease, and reduction of the risk of cancer (Donaldson 2004, Kong et al. 2003, Kowalczyk et al. 2003, Lila 2004b, Stintzing and Carle 2004). They also enhance the immune function by boosting cytokines production. Anthocyanins or anthocyanidins can enhance the tumor necrosis factor  $\alpha$  (TNF- $\alpha$ ) production in macrophages, which is requisite for their engagement in scavenging (Jian and Mazza 2002).

#### ***1.6.1. Protection of DNA by preventing radical hydroxyl attack***

Cyanidin-DNA co-pigmentation is hypothesized to be the mechanism that prevents DNA from oxidative damage (Sarma and Sharma 1999, Webb et al. 2008). The binding of anthocyanins to DNA protects both from oxidative damage. It has also been

suggested that anthocyanins can stabilize the DNA triple-helical complex (Mas et al. 2000). A study of hordeumin, a high-molecular weight condensed tannin-like purple pigment, revealed that it possesses antimutagenicity effect. Hordeumin can also decrease the reverse mutation of dimethyl sulfoxide extracts from grilled beef (Deguchi et al. 2000b, Deguchi et al. 2000a).

#### ***1.6.2. Improvement of night vision and near-sightedness***

When black currant juice extract is included in a human diet, a remarkable improvement of visual adaptation results (Matsumoto et al. 2001). Anthocyanins have been suggested as remedies for vision disorders, in enhancing visual acuity, and increasing capillary resistance (Lila 2004b). Retinopathy and cataracts, which are caused by diabetes mellitus, can be remedied by ingestion of anthocyanins. Anthocyanins can inhibit the  $\alpha$ -glucosidase enzyme (AGH), a glucose cleavage enzyme located in the epithelium of the small intestine, thereby reducing excess glucose absorption (Matsui et al. 2001a, Matsui et al. 2001b). The acylated forms of anthocyanins exhibit better capability for AGH inhibition.

#### ***1.6.3 Protection against neurological disorders***

Rats which are fed with an anthocyanin-rich diet demonstrated improvement in their motor and cognitive functions and reversed age related decrements (Joseph et al. 1999, Youdim et al. 2000a). The antioxidant activity and bioavailability of anthocyanins in endothelial cells helped prevent atherosclerosis and neurodegenerative disorders. Research also shows that cyanidin-3,5-diglucoside and cyanidin-3-sambubioside-5-glucoside enhance the resistance of blood cell to oxidative stress (Youdim et al. 2002, Youdim et al. 2004, Youdim et al. 2000b).

#### ***1.6.4. Prevention of cardiovascular disease***

The rich anthocyanin pigments in red wine has been postulated to be a contributing factor behind the ability of red wine to help prevent cardiovascular disease. Activities such as antithrombotic potential, reactive oxygen species scavenging, lipoprotein oxidation inhibition, platelet function inhibition, and nitric oxide release enhancement of anthocyanins in red wine or grape juice may help to prevent heart attack (Lila 2004a, Lila 2004b, Stintzing and Carle 2004). Anthocyanins are a strong inhibitor of nitrated tyrosine *in vitro*, and an inhibitor of vascular endothelial growth factor expression in vascular smooth muscle cells; therefore they can prevent atherosclerotic lesions in human coronary arteries (Tsuda et al. 2000). Bioflavonoid mixtures which also contain anthocyanins can accelerate ethanol metabolism, reduce inflammatory and edematic symptoms, and improve permeability and strength of capillaries (Lila 2004a, Lila 2004b, Stintzing and Carle 2004). Pure anthocyanins are seven times more effective than  $\alpha$ -tocopherol as antioxidant and inhibit lipid peroxidation (Lila 2004b).

#### ***1.6.5. Reduction in the risk of cancer***

As described above, anthocyanins can prevent DNA oxidative damage and reduce mutagenicity. They can also inhibit the cyclooxygenase enzyme, which is a marker for the initiation stage of carcinogenesis (Donaldson 2004, Kong et al. 2003, Kowalczyk et al. 2003, Lila 2004b, Stintzing and Carle 2004). Anthocyanins from cherry can inhibit the growth of human colon cancer cell lines. Mice fed with a cherry diet have been found to have fewer and smaller adenomas compared with the control group (Kang et al. 2003). Anthocyanins and related flavonoids can inhibit not only the initiation stage but also the promotion and proliferation stage of chemically induced carcinogenesis (Donaldson 2004, Lila 2004b). Cyanidin and delphinidin are the

groups of anthocyanins which provide stronger activity of human tumor cell growth inhibition by shutting off downstream signaling cascades (Meiers et al. 2001).

## **1.7. CONCLUSION**

Because of the multiple benefits of anthocyanins to human health, anthocyanins are now considered not only healthy compounds in food, but also potential medicines. The antioxidant activity of anthocyanins is higher than that of vitamin E ( $\alpha$ -tocopherol), ascorbic acid and  $\beta$ -carotene (Kong et al. 2003, Stintzing and Carle 2004). Anthocyanins also help decrease the amount of oxidized LDL, inhibit adhesion and reaction of leucocytes, provide antibacterial properties, decrease prostaglandin level, and inhibit the activity of enzymes inducing apoptosis etc (Kong et al. 2003, Kowalczyk et al. 2003). The broad functions of anthocyanins in plants and human health have caught the attention of the general public. However, the mechanism through which the transcription factors that help results anthocyanin synthesis in plants and the role of anthocyanins in helping to prevent certain human disease are still not well understood, and require further studies.



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## CHAPTER 2

### TRANSCRIPTIONAL REGULATION OF ANTHOCYANIN BIOSYNTHESIS IN PURPLE CAULIFLOWER MUTANT

#### 2.1. ABSTRACT

Anthocyanins are the largest group of water-soluble pigments in the plant kingdom, and are responsible for the blue, red, and purple colors of many fruits, vegetables, grains, flowers, and leaves. In recent years, numerous studies have suggested that anthocyanins serve as antioxidants in the diet that can reduce the risk of a number of human diseases. To gain a better understanding of the regulation of anthocyanin biosynthesis in plants, we studied a purple cauliflower mutant that exhibited a distinctive purple coloration in the curd and young leaves. Biochemical characterization of the mutant showed that cyanidin 3-(coumaryl-caffeyl)glucoside-5-(malonyl)glucoside was the predominant anthocyanins identified in the purple curd tissue and that it accumulated to a level of approximately 3.75 mg of cyanidin equivalent per gram of fresh weight. Microscopic analysis of the mutant revealed that the anthocyanins were accumulated primarily in the central cells of the curd tissue and in the sub-epidermis of the young leaves. Examination of the transcript levels of the anthocyanin biosynthetic genes and related transcription factors by Northern blot and RT-PCR analysis indicated that while most of the genes examined showed similar levels of expression, the structural genes encoding the enzymes flavonoid 3'-hydroxylase (F3'H), dihydroflavonol reductase (DFR), and leucoanthocyanidin dioxygenase (LDOX), as well as the transcription factors *BoP2* and *BoTT8*, were expressed highly in both the curd and leaf tissues of the purple mutant in comparison with those in wild type cauliflower. Preliminary genetic segregation study of the F<sub>2</sub> plants suggested that the purple mutation is controlled by a single semi-dominant gene.

## 2.2. INTRODUCTION

Vegetables and fruits are fundamental components of the human diets. They not only are important sources of essential vitamins and minerals, but also contain a wide variety of secondary metabolites essential to human health. Colored vegetables and fruits have recently been gaining popularity as functional foods, owing to their high levels of plant pigments with potent nutritional and health-promoting effects (Lila 2004, Lila 2009).

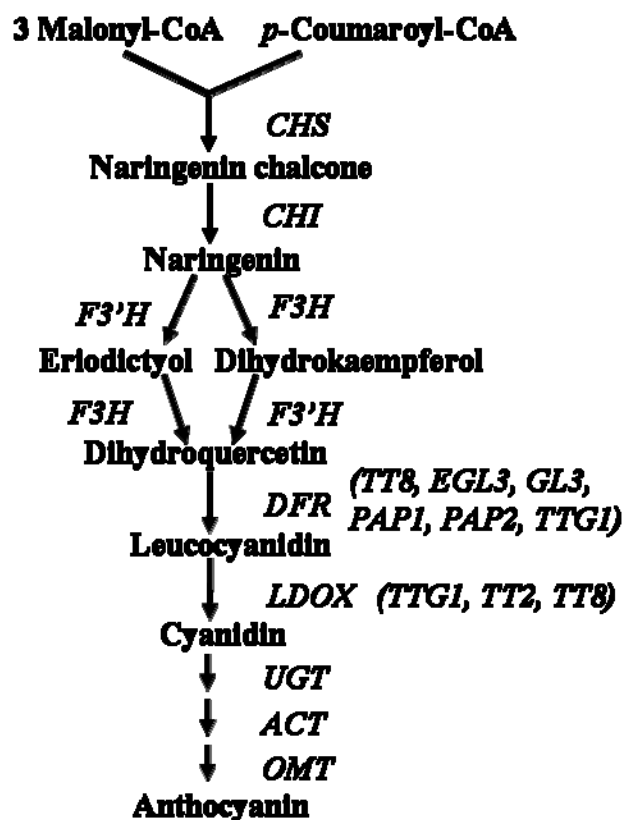
Purple cauliflower (*Brassica oleracea* L var. *botrytis*) is a very eye-catching vegetable that originates from Europe. It exhibits a striking purple color in the curd tissue, and the color appears to be completely independent of the temperature. Several purple cauliflower varieties are available commercially, including Graffiti, Purple Cape, Violet Queen, Violetta Italia and Rosalind (Reimer Seeds, NC, USA; West Coast Seeds, BC, Canada). The purple coloration is the result of the accumulation of anthocyanins.

Anthocyanins are the most prominent class of flavonoids and responsible for the blue, red, and purple colors of many fruits, vegetables, grains, flowers, and leaves. They have been postulated to bring significant health benefits to humans (Lila 2004, Lila 2009). High dietary intake of anthocyanin-rich foods is associated with reduced risk of cancers (Cooke et al. 2005, Fimognari et al. 2008, Hou 2003, Thomasset et al. 2009), prevention of cardiovascular diseases (Lazze et al. 2006, Youdim et al. 2000), improvement of night vision and near-sightedness (Lee et al. 2005, Matsumoto et al. 2003), and protection against neurological disorders (Cho et al. 2003, Kang et al. 2006).

Several kinds of anthocyanins exist in the plant kingdom (Deroles 2009, Tanaka et al. 2008). The first committed step in the cyanidin biosynthetic pathway is the condensation of three molecules of malonyl-CoA with one molecule of *p*-

coumaroyl-CoA by chalcone synthase (CHS) to produce yellow chalcone (Figure 2.1). The chalcone is subsequently isomerized by chalcone isomerase (CHI) to prototype pigment naringenin. Hydroxylation of naringenin at the 3' position by flavanone 3-hydroxylase (F3H) yields dihydrokaempferol. Dihydrokaempferol were then added a hydroxyl groups at the 3' positions by flavonoids 3'-hydroxylase (F3'H) to form dihydroquercetin. Dihydroquercetins undergo a reduction reaction with dihydroflavonol 4-reductase (DFR) to form colorless leucoanthocyanidins. The leucoanthocyanidins are oxidized by leucoanthocyanidin dioxygenase (LDOX) into the corresponding cyanidins. Methylation, glycolation, and/or acylation of the different anthocyanidins by *O*-methyltransferase (OMT), UDP-glucosyltransferase (UGT), and acyltransferase (ACT) result in the formation of anthocyanins with increased stability and water solubility (Ben-Meir et al. 2002, Springob et al. 2003, Tanaka et al. 2008). The synthesized anthocyanins in the cytosol are then sequestered into the vacuole for storage (Goodman et al. 2004).

Numerous anthocyanin regulatory genes have been identified from researching *Arabidopsis*, maize, petunia, snapdragon, and other plant species (Allan et al. 2008, Broun 2005, Koes et al. 2005, Mol et al. 1998). R2R3 MYB and basic helix-loop-helix (bHLH) transcription factors, as well as WD40, proteins represent the three major families of anthocyanin regulatory proteins. They form a so called WBM regulatory complex in activating the expression of anthocyanin structural genes (Allan et al. 2008, Gonzalez et al. 2008, Lepiniec et al. 2006, Vom Endt et al. 2002). In *Arabidopsis*, several R2R3 MYB proteins, such as Production of Anthocyanin Pigment 1 (PAP1), PAP2, MYB113, MYB114, MYB4 and MYBL2 (Borevitz et al. 2000, Dubos et al. 2008, Gonzalez et al. 2008, Hemm et al. 2001, Matsui et al. 2008), three bHLH proteins, Transparent Testa 8 (TT8) (Nesi et al. 2000), Glabra 3 (GL3), and Enhancer of Glabra 3 (EGL3) (Payne et al. 2000, Zhang et al. 2003), and the WD40 repeat



**Figure 2.1.** Cyanidin biosynthetic pathway. Regulatory genes affecting DFR and LDOX expression are listed in parentheses. PAL, phenylalanine ammonia lyase; C4H, cinnamate 4-hydroxylase; 4CL, 4-coumarate CoA ligase; CHS, chalcone synthase; CHI, chalcone isomerase; F3H, flavanone 3-hydroxylase; F3'H, flavonoid 3'-hydroxylase; DFR, dihydroflavonol 4-reductase; LDOX, leucoanthocyanidin dioxygenase; FLS, flavonol synthase; UGT, UDP-glucosyltransferase; ACT, acyltransferase; OMT, O-malonyltransferase; TT8, transparent testa 8; EGL3, enhancer of glabra 3; GL3, glabra 3; PAP1, production of anthocyanin pigment 1; PAP2, production of anthocyanin pigment 2; TTG1, transparent testa glabra 1; TT2, transparent testa 2.



protein Transparent Testa Glabra 1 (TTG1) (Walker et al. 1999) are involved in anthocyanin biosynthesis. Different combinations of these transcription factors in the transcriptional complex result in tissue specific regulation of the expression of structural genes involved in anthocyanin synthesis (Gonzalez et al. 2008). Furthermore, the WBM complex not only regulates the expression of structural genes, but also can regulate the expression of transcription factors in the complex (Baudry et al. 2006, Espley et al. 2009). Although these transcription factors have similar functions as those in maize, they differ in their control of a subset of anthocyanin biosynthesis genes (Quattrocchio et al. 1993). Thus, transcription factors exhibit species-specific differences in the regulation of expression of anthocyanin biosynthetic genes.

Although there are many recent studies on the understanding of the regulation of anthocyanin biosynthesis in flowers, fruits, staple crops and model plants, only a few have focused on vegetables (Park et al. 2008, Yuan et al. 2009). Cauliflower is the most closely related cultivated crop genus to *Arabidopsis* and they typically share over 85% nucleotide sequence identity in the coding regions (Babula et al. 2003, Lan et al. 2000). The fully sequenced genome of *Arabidopsis* facilitates the cloning of genes involved in anthocyanin production from cauliflower to study anthocyanin regulation in cauliflower. The purple cauliflower mutant Graffiti exhibits an interesting tissue specific anthocyanin accumulation phenotype and produces striking purple color in the curd and seed endosperm, the tissues with agriculture importance. Thus, the purple cauliflower mutant serves as a good model for studying anthocyanin biosynthesis in vegetables.

In order to gain a better understanding of the regulatory mechanism of anthocyanin biosynthesis in cauliflower, we examined the expression of anthocyanin biosynthetic structural genes as well as regulatory genes between the wild type cauliflower, Stovepipe, and the purple mutant, Graffiti, together with a phenotypic and

biochemical characterization of the mutant. Gene expression analysis revealed that several genes late in the anthocyanin biosynthetic pathway, including *BoF3'H*, *BoDFR*, and *BoLDOX*, and several transcription factors, such as the MYB transcription factor, *BoP2*, and the bHLH transcription factor, *BoTT8*, are differentially expressed between the purple mutant and wild type cauliflower. We have identified cyanidin 3-(coumaryl-caffeyl)glucoside-5-(malonyl) glucoside as the major anthocyanin accumulated in Graffiti. Genetic segregation showed that the mutation is controlled by a single, semi-dominant gene.

## **2.3. MATERIALS AND METHODS**

### ***2.3.1. Plant materials and growth conditions***

The cauliflower (*Brassica Oleracea* L. Var. *Botrytis*) cultivars used for this study included a commercial purple cauliflower cultivar, Graffiti (Harris Seeds, Rochester, NY) and a white cultivar, Stovepipe, serving as the wild type control. A F<sub>2</sub> population of 102 individuals was generated from a cross between the Graffiti and Stovepipe, and then by selfing the heterozygous F<sub>1</sub> plants. The genotypes of the F<sub>2</sub> individuals were visually determined based on purple pigmentation and further confirmed in some cases by analyzing 16 F<sub>3</sub> plants.

The cauliflower plants were grown in a greenhouse under a 14-h-light/10-h-dark cycle at 23°C. Samples of the curds, young and old leaves, petals, young flower buds, roots, and young and old siliques from each cultivar were collected, frozen in liquid nitrogen, and stored at -80°C until use. The newly emerged young leaves were collected when they were about 4 to 6 cm<sup>2</sup> in area. Old leaves were collected when they were over 40 cm<sup>2</sup> in area. A young flower bud is defined as one whose diameter did not exceed 0.5 cm. Flower tissues were harvested one day after they had fully

blossomed. Young and old siliques are defined as those whose lengths were around 2cm and 4cm, respectively.

### ***2.3.2. HPLC-ESI-MS/MS analysis of anthocyanins***

The anthocyanins in Graffiti were extracted and analyzed by HPLC following a previously described method (Wu and Prior 2005). The fresh curd sample was freeze-dried. The dried sample (0.5 g) was then grounded into powder and extracted twice in total 25 mL methanol/H<sub>2</sub>O/acetic acid (85:15:0.5). The extract was diluted 2 fold, and aliquot (10 µl) was injected into the Zorbax Stablebond Analytical SB-C18 column (4.6 x 250 mm, 5 µm, Agilent Technologies, Rising Sun, MD) and separated using 5% formic acid (A) and 100% methanol (B) as mobile phases. Quantification was carried out based on peak areas and a calibration curve which was generated with a commercial standard of cyanin 3,5-diglucoside chloride (INDOFINE Chemical Company, Hillsborough, NJ).

In collaboration with Dr. Ronald Prior at the Arkansas Children's Nutrition Center, the composition of anthocyanins in the purple cauliflower curd was identified using HPLC-ESI-MS/MS. Low-resolution electrospray mass spectrometry as previously described (Wu and Prior 2005) was performed to identify the major individual anthocyanin peaks in the purple curd sample.

### ***2.3.3. Light microscopy***

Fresh cauliflower young leaves and curds were hand sectioned using double edged razor blades under a stereomicroscope. The samples were then mounted in water under cover slips, and the images were examined with an Olympus BX60 microscope and recorded as previously described (Li et al. 2001).

#### 2.3.4. RNA analysis

Total RNA samples from the purple and white cauliflower tissues were extracted using Trizol reagent following the instructions of the manufacturer (Invitrogen, Carlsbad, CA). The mRNA was isolated from total RNA samples using the PolyAtract® mRNA isolation system IV (Promega, Madison, WI).

For Northern blot analysis, mRNA (2 µg) samples were separated on formaldehyde agarose gels and transferred onto the Hybond N<sup>+</sup> filters (Amersham, Piscataway, NJ). Equal loading of mRNA was confirmed by ethidium bromide staining on the agarose gels. The filters were first prehybridized in the ULTRAhyb (Ambion, Austin, TX) solution at 42°C for 30 min, and then hybridized overnight with a [<sup>32</sup>P]dATP labeled probe as described previously (Li et al. 2001). The probes used included *CHS* (U21762), *CHI* (U20894), *F3H* (U14735), and *LDOX* (YAY780) from *Arabidopsis* gene clones (TAIR, [www.arabidopsis.org](http://www.arabidopsis.org)) as well as *F3'H* (7 and 8), *DFR* (9 and 10), *UGT* (13 and 14), and *Actin* (17 and 18) (Table A1.1) amplified from white cauliflower DNA using specific primers. The filters were washed in 2× SSC at 42°C for 5 min twice, and in 0.1× SSC with 0.1% (w/v) SDS for 15 min twice.

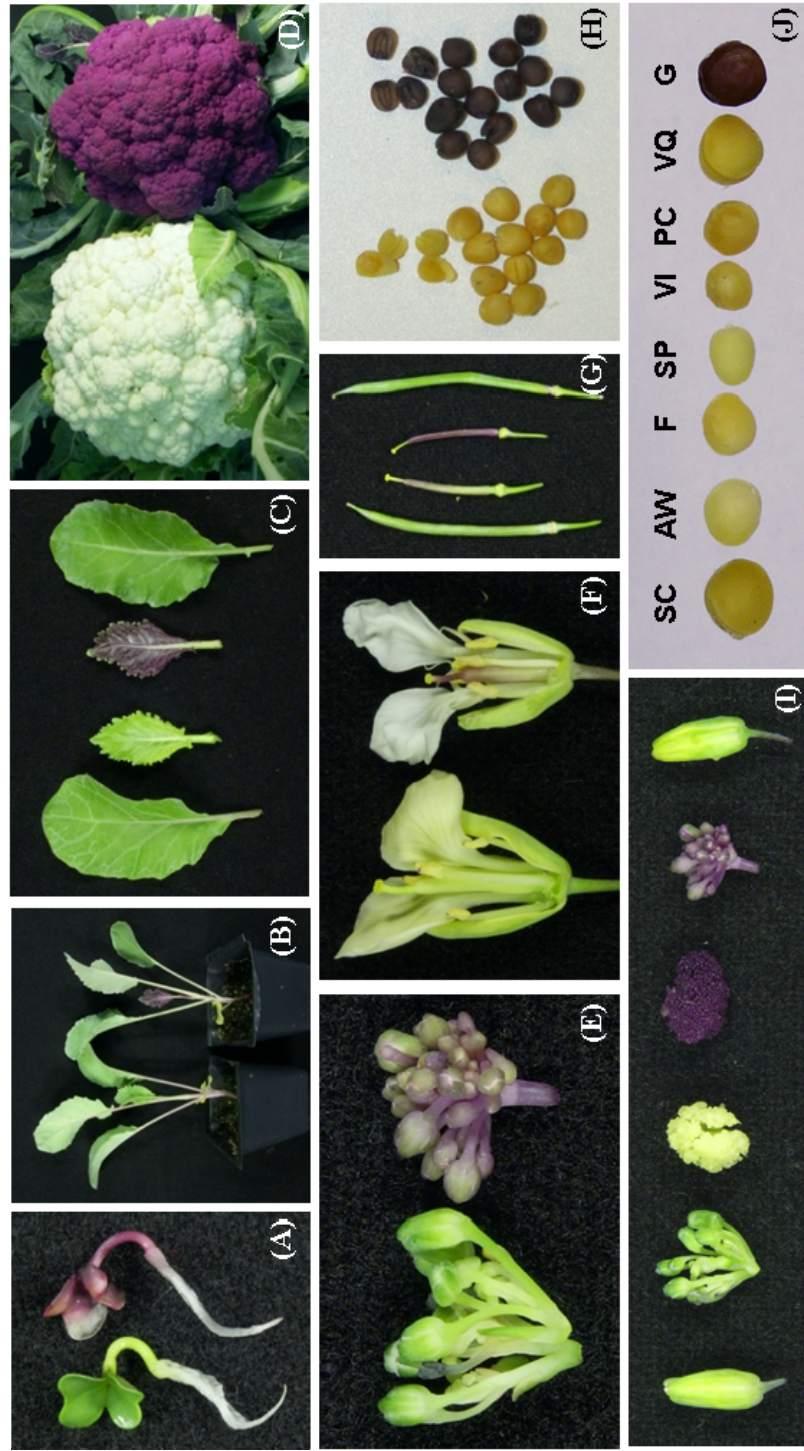
For semi-quantitative RT-PCR analysis, cDNA was synthesized from the total RNA (5µg) using the Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA). The cDNA concentrations in different samples were normalized based on the amplification of *BoAct1* (Lu et al. 2006). Gene specific primers were designed based on the sequences in both the *Arabidopsis* (TAIR) and *Brassica Oleracea* (TIGR) databases (Yuan et al. 2009). Gene specific primers of *BoCHS* (1 and 2), *BoCHI* (3 and 4), *BoF3H* (5 and 6), *BoF3'H* (7 and 8), *BoDFR* (9 and 10), *BoLDOX* (11 and 12), *BoTT2* (25 and 26), *BoEGL3* (33 and 34), *BoFUS3* (27 and 28), *BoTTG2* (21 and 22), *BoANL2* (31 and 32), *BoRNS1* (29 and 30), *BoTTG1* (19 and 20), *BoTT8* (23 and 24),

*BoP1* (35 and 36), *BoP2* (37 and 38), *BoP3* (39 and 40) and *BoP4* (41 and 42) (Table A1.1) were used to amplify the target genes with optimized cycles of amplification.

## **2.4. RESULTS**

### ***2.4.1. Phenotypic characterization of the purple cauliflower***

The purple cauliflower arose as the result of a spontaneous mutation found in a cauliflower field about 20 years ago. The purple hue appears to be independent of the temperature, but is intensified by high light. The purple cauliflower mutant grows and develops similarly to the white (wild type) cauliflower. Intense purple coloration in the purple cauliflower mutant was observed in several tissues of the plant (Figure 2.2), including the newly germinated seedlings (A), the very young leaves (B and C), the curds (D), the very young flower buds (E and I), young siliques (G), and the endosperms of seeds (H). The purple color was not seen in the roots (A), older leaves (B and C), stems (B), flowers (F), and older siliques (G). The purple phenotype appears to be associated with very young tissues, curds, and seeds, rather than with flowers, the most common anthocyanin-accumulating tissue. Under the same growth conditions, the wild type cauliflower plants exhibit no observed purple hue in those affected tissues (Figure 2.2). There exist other purple cauliflower cultivars such as Violet Queen (VQ), Violetta Italia (VI) and Purple Cape (PC) (Reimer Seeds, Mount Holly, NC), which are commercially available. Although they have the purple curd phenotype, the endosperms of their seeds have normal coloration, like those in the white cultivars (Figure 2.2.J). Thus, the purple mutant studied exhibits a unique tissue-specific pattern of anthocyanin accumulation.



**Figure 2.2.** Phenotypic comparison between the wild type cauliflower and the purple mutant. Cauliflower (A) young seedlings, (B) young plants, (C) young (inner) and old leaves, (D) curds, (E) young flower buds, (F) flowers, (G) young (inner) and old siliques, (H) endosperms, and (I) different floral developmental stages of white cultivar Stovepipe (left) and purple cultivar Graffiti (right). Graffiti is the only purple cauliflower cultivar with anthocyanin accumulation in the seeds (J). White cultivar: Snow Crown (SC), Absolute White (AW), Fremont (F) Stovepipe (SP), Purple Italia (VI), Purple Cape (PC), violet queen (VQ) and Graffiti (G)

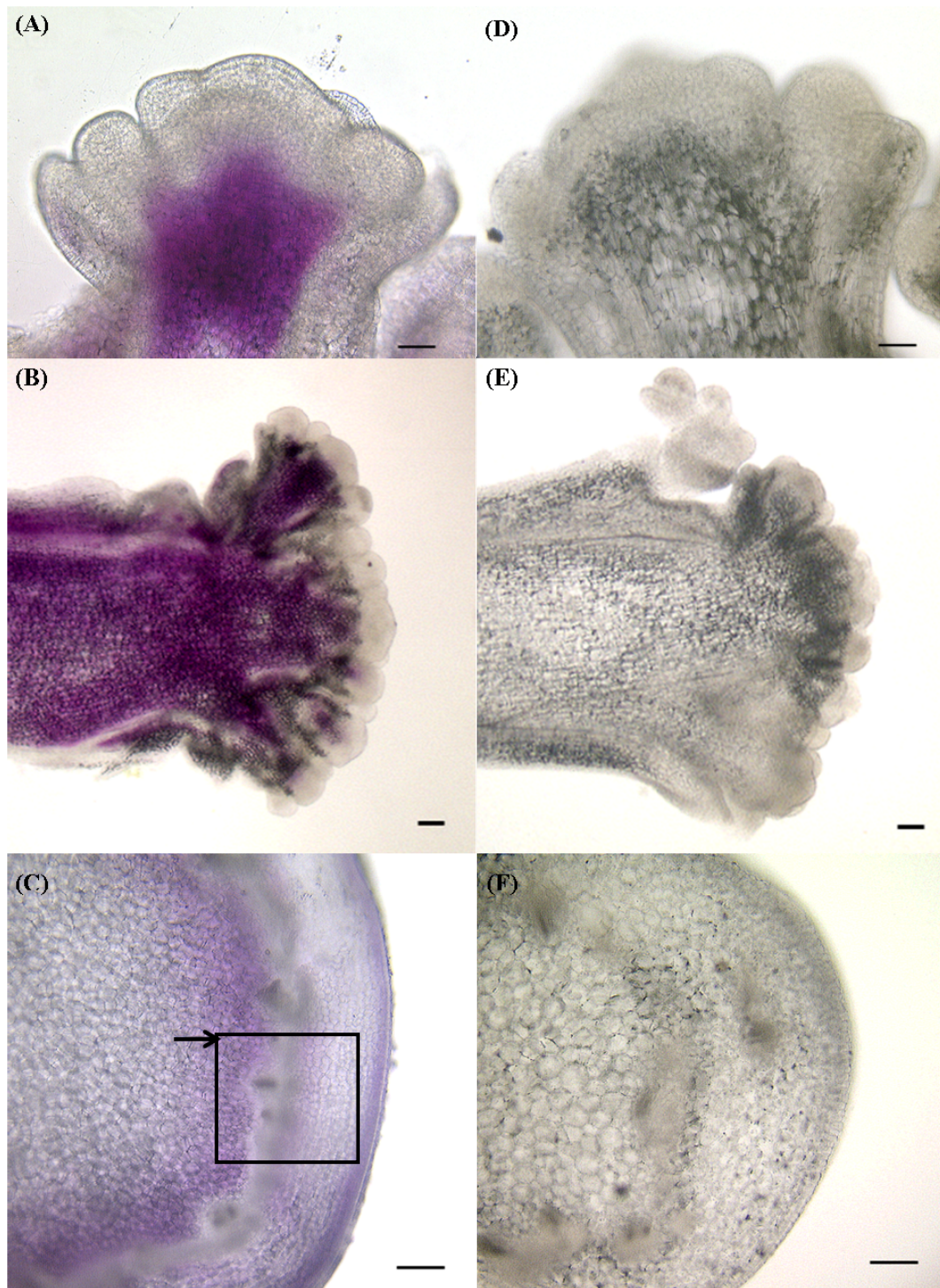
#### ***2.4.2. Cytological characterization of the purple cauliflower Graffiti***

To obtain more detailed information on the distribution of anthocyanins in cells of the tissues affected, light microscopy analysis of freehand sections was performed. Since anthocyanins are water soluble compounds, fixation is not applicable to the plant tissue. In the curd tissue, the purple color was primarily localized in the center of the branches rather than in the inflorescent meristematic cells in the outer layers of the curd tissue (Figure 2.3.A). The prominent purple color was extended to more differentiated cells in the curd tissue (Figure 2.3.B). In the base of the curd branch, the anthocyanins were accumulated closer to the vascular cambium and the outer layer of cortex (Figure 2.3.C, C1). In the young leaf tissue, the anthocyanins were accumulated in the outer layer of sub-epidermal cells on both sides of leaves (Figure 2.4.A) and appeared to be concentrated in the vacuoles (Figure 2.4.B, B1). No anthocyanin accumulation was found in the corresponding cells in the tissues of wild type plants (Figure 2.3.D-F, and Figure 2.4.C-D).

#### ***2.4.3. Purple mutant contains predominantly cyanidin glucoside***

To examine the composition and content of the anthocyanins accumulated in the purple mutant, we performed HPLC analysis on the samples of young leaf, curd and seed tissues. As expected, the leaves, curds and seeds of the wild type control plants contained undetectable quantities of anthocyanins, indicating that the anthocyanin pathway was biochemically quiescent in these tissues under normal growth conditions. In contrast, high levels of anthocyanins were found in tissues of the purple cauliflower mutant (Figure 2.5.A-C). The accumulation level in the curds was approximately 3.75 mg cyanidin g<sup>-1</sup> of fresh weight, a level which was higher than those found in blueberries, which ranged from 1.1 to 2.6 mg g<sup>-1</sup> of fresh weight (Gao and Mazza 1994). The accumulation levels of anthocyanins in the leaves and seeds were 1.06 and



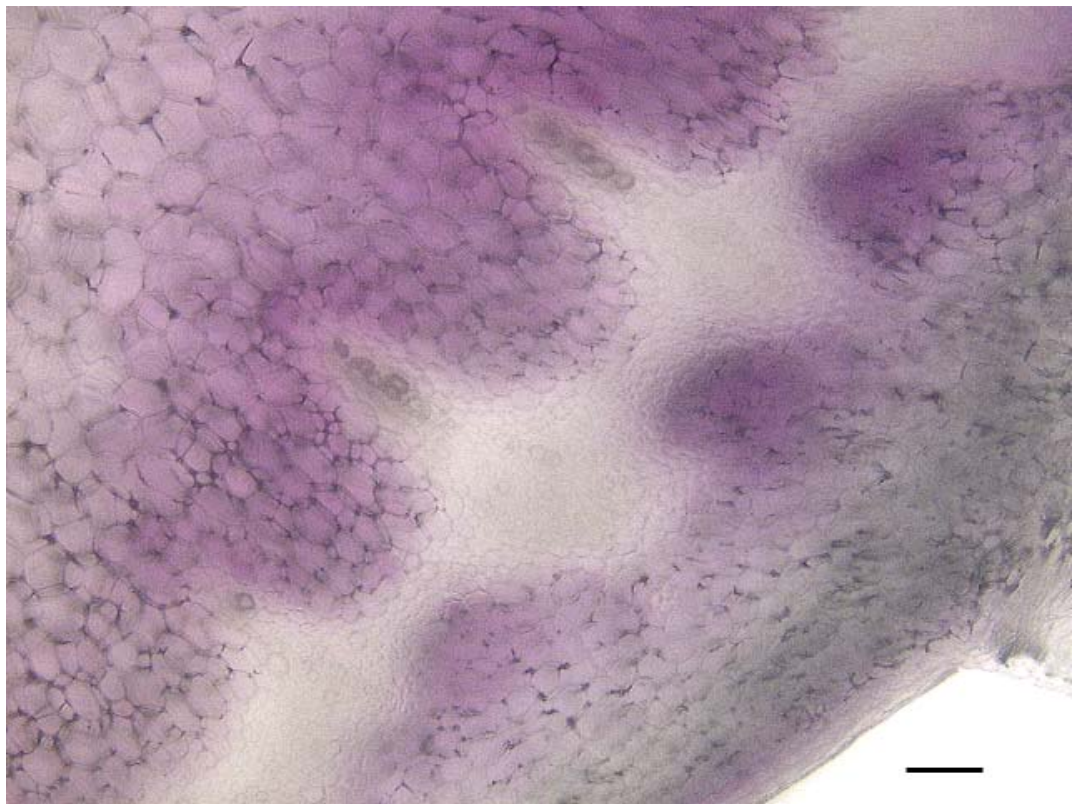


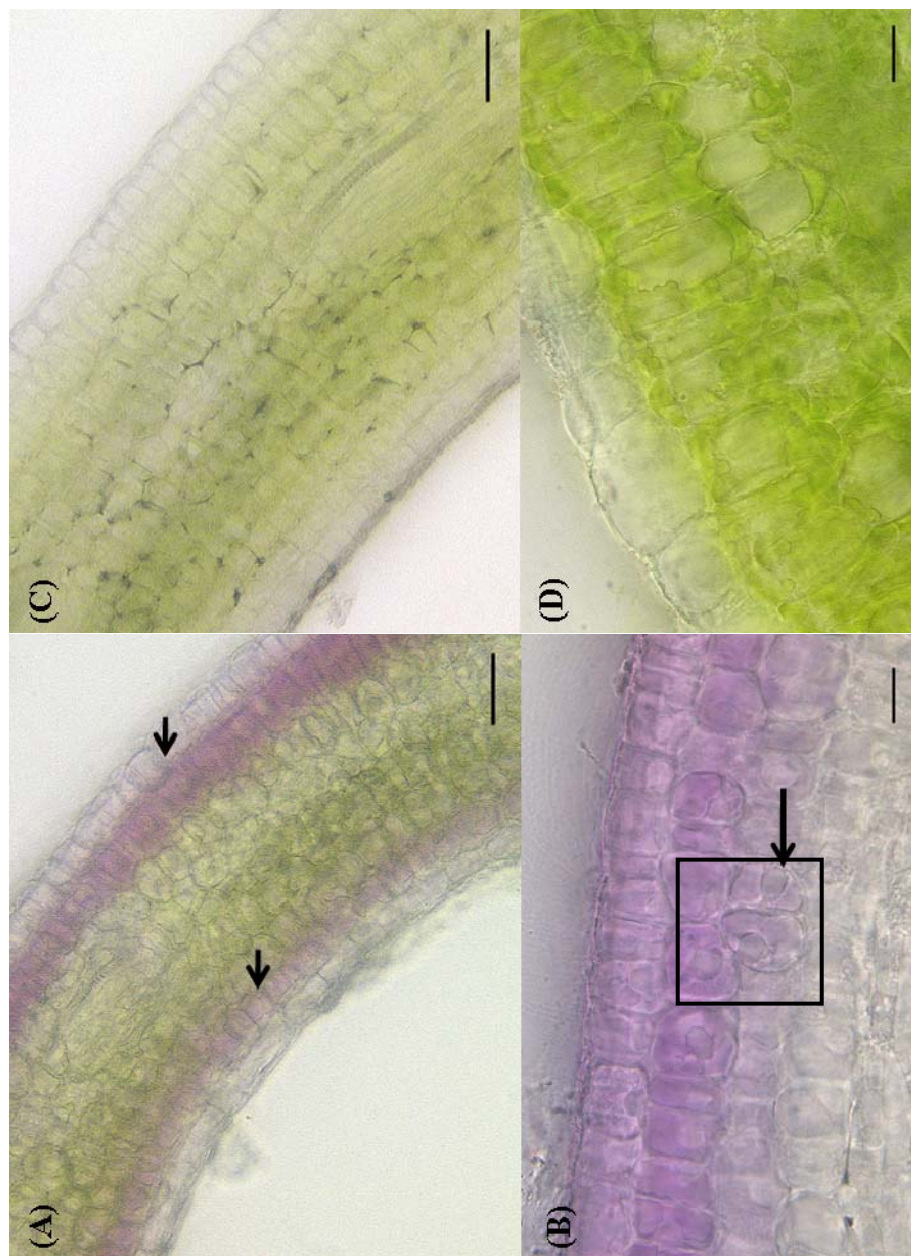
**Figure 2.3.** Anthocyanin accumulation in the curd tissue of purple cauliflower Graffiti (A-C, C1) compared to white cauliflower Stovepipe (D-F). Accumulation occurred in the central region of curd meristem tissue (A and B) and near the vascular bundle (C). Scale bars: A, C1 and D, 50  $\mu\text{m}$ ; B, C, E, F, 100  $\mu\text{m}$ .



(C1)

**Figure 2.3.** continued

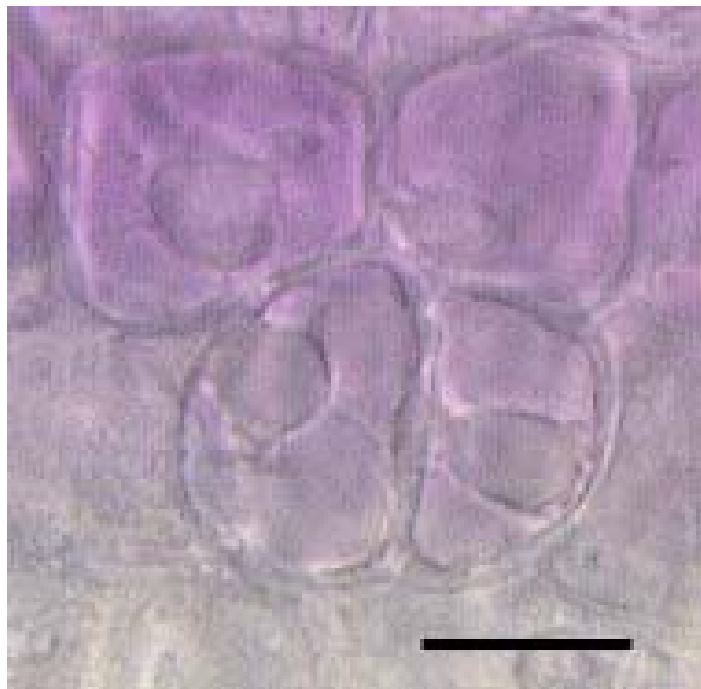


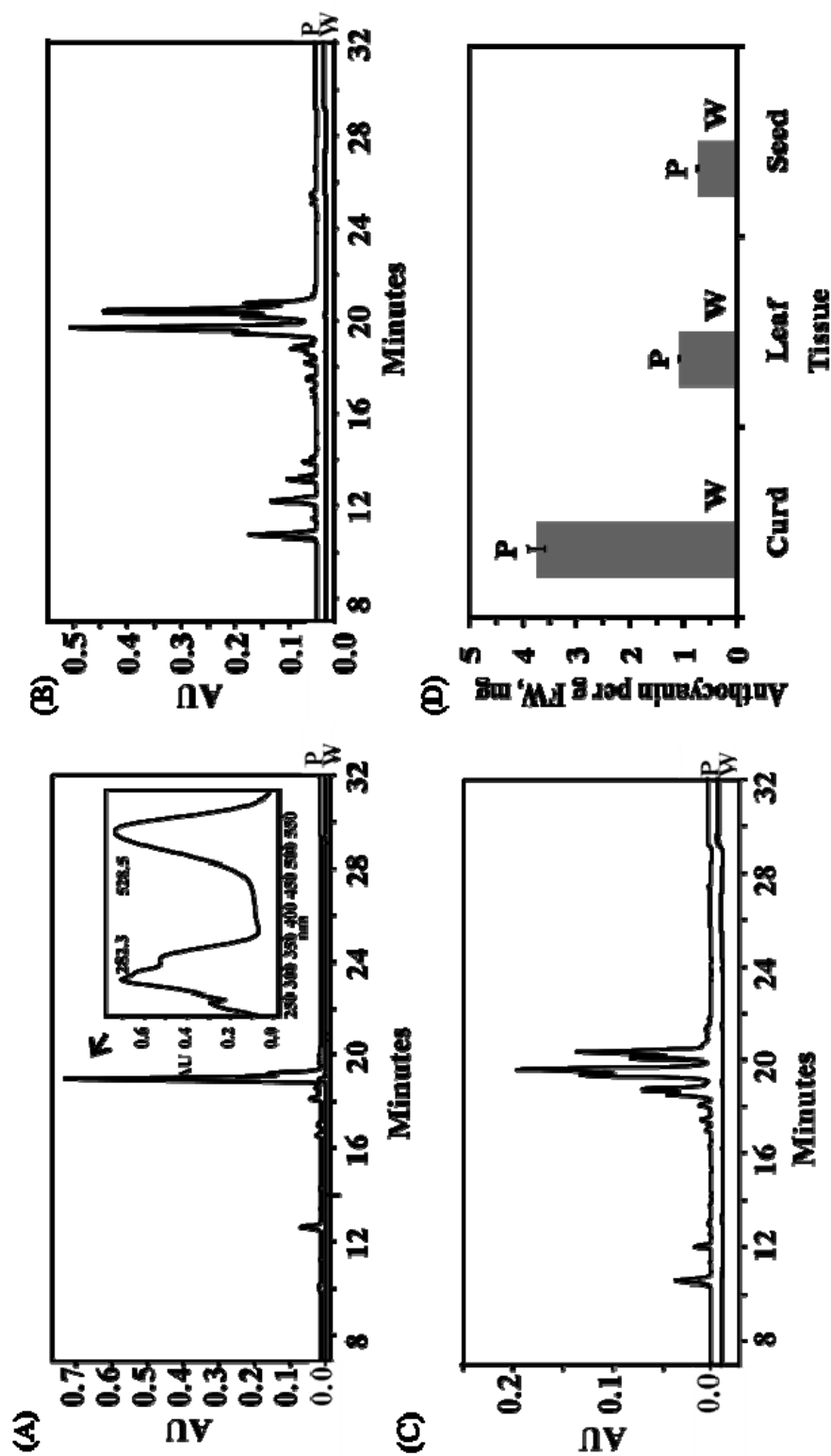


**Figure 2.4.** Anthocyanin accumulation in the leaf tissue of purple cauliflower Graffiti (**A and B**) compared to white cauliflower Stovepipe (**C and D**). Accumulation occurred in the vacuole (**B, B1**) of the sub-epidermal cells on both sides of the leaves (**A**). Scale bars: A and C, 30  $\mu\text{m}$ ; B, B1 and D, 10  $\mu\text{m}$ .

**(B1)**

**Figure 2.4.** continued





**Figure 2.5.** HPLC elution profiles of anthocyanins accumulated in the (A) curd, (B) leaf and (C) seed tissues of purple cauliflower (P) compared to white cauliflower (W) at A520. The accumulation levels of anthocyanins in the curd, leaf and seed tissue of purple cauliflower are approximately 3.75, 1.06, and 0.74 mg/g of fresh weight, respectively (D).

0.74 mg g<sup>-1</sup> of fresh weight, respectively (Figure 2.5.D).

There are several different groups of anthocyanins in the plant kingdom (Deroles 2009, Tanaka et al. 2008). To identify the anthocyanin composition in the purple mutant, anthocyanins in the curd tissues were separated and analyzed using HPLC-ESI-MS/MS by Dr. Ronald Prior at the Arkansas Children's Nutrition Center. As shown in Figure 2.5.A, the curd tissue of the purple mutant contained one major peak (Figure 2.6.A). The major peak identified was cyanidin 3-(coumaryl-caffeyl) glucoside-5-(malonyl)glucoside, while the other minor peaks were different forms of cyanidin glycosides (Figure 2.6.B). Although the same major peak was observed for the leaves and seeds tissues, the fact that the other minor peaks increased dramatically hinted that the residue modification of the major cyanidin occurs in other tissues (Figure 2.5.B-C)

#### ***2.4.4. The purple mutation was controlled by a single semi-dominant gene***

To determine whether the purple cauliflower mutant is controlled by a single or multiple genes, we crossed the purple cauliflower mutant Graffiti to an inbreeding white cauliflower cultivar Stovepipe, selfed the heterozygous F<sub>1</sub> plants, and generated a large F<sub>2</sub> population. A subpopulation of 102 individuals was germinated in a greenhouse and the genotype of these F<sub>2</sub> individuals was identified by visually examining the presence or absence of purple color in very young leaves and curds of the progeny, followed by further confirmation in some cases with 16 F<sub>3</sub> individuals. The segregation ratio of these F<sub>2</sub> plants for white: light purple: dark purple was 31:53:18. Chi-square test yielded a good fit with 1:2:1 (P> 0.05), which is consistent with the ratio expected for the progeny derived from selfing a parent heterozygous at one locus. This result suggests that the purple phenotype is controlled by a single,

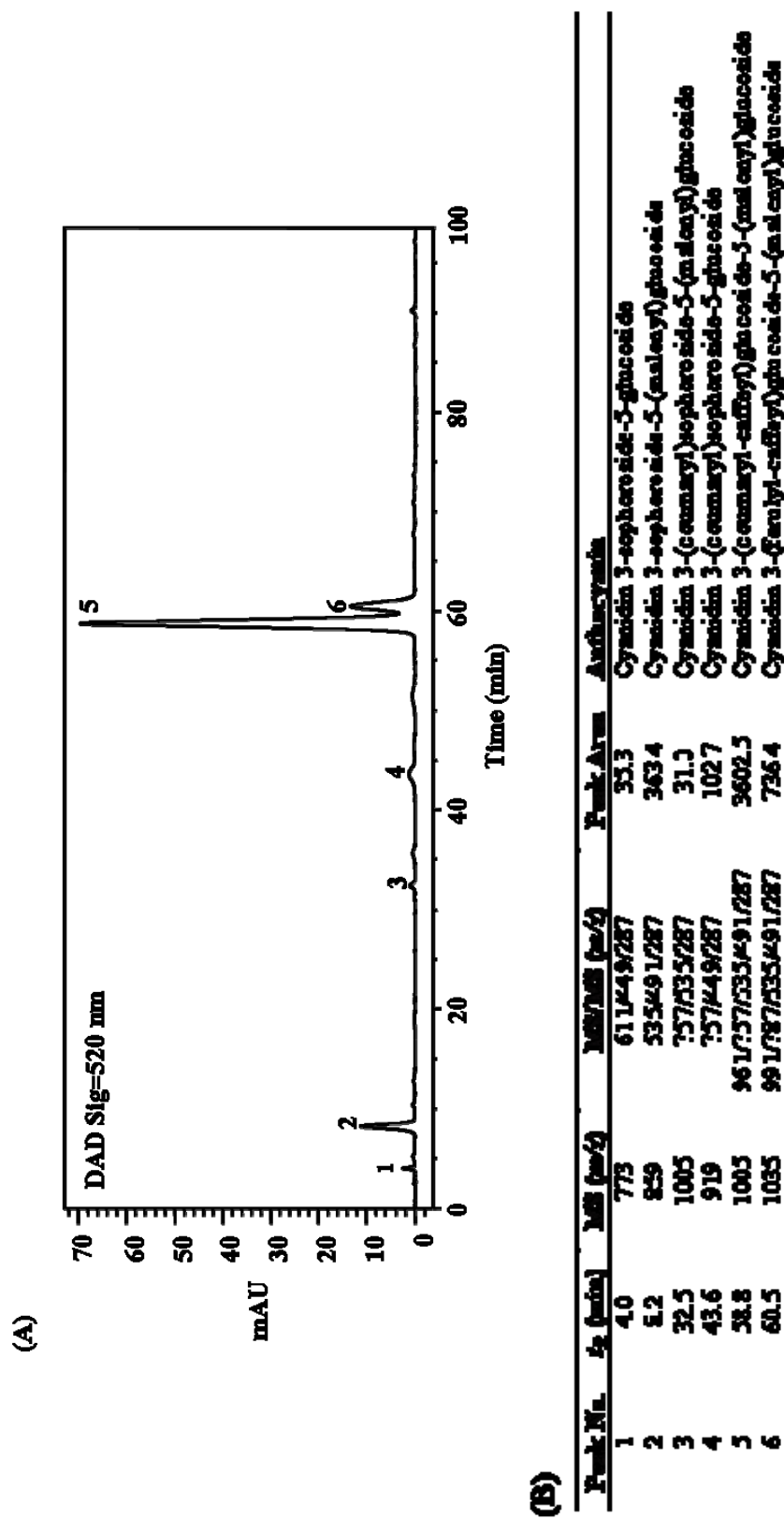
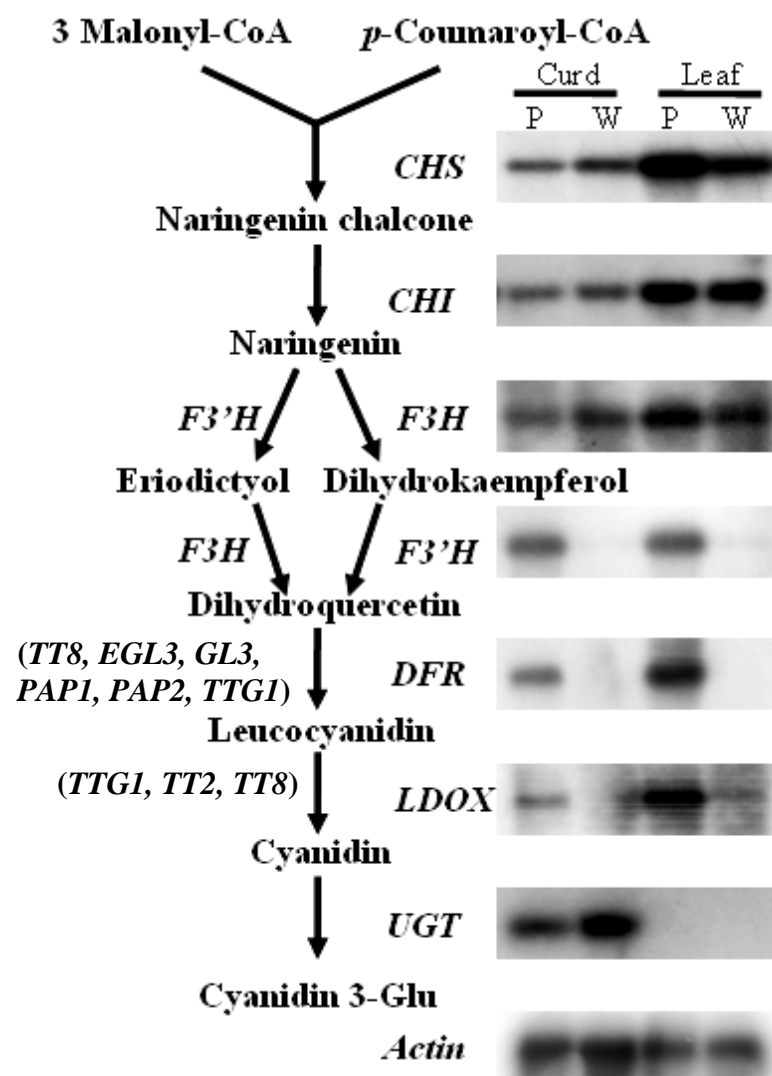


Figure 2.6. Anthocyanin composition in purple cauliflower Graffiti. (A) Typical HPLC elution profile of anthocyanins from curd tissue. (B) Anthocyanin compositions of the major peaks.

semi-dominant gene. Thus, we designate the symbol *pr* for the *Purple* gene, and *Pr* for its wild type counterpart.

#### **2.4.5. Expression of anthocyanin biosynthetic genes**

Transcriptional regulation of structural genes appears to be the major mechanism by which the anthocyanin biosynthesis is regulated in plants (Koes et al. 2005, Vom Endt et al. 2002). All the major anthocyanin structural genes have been isolated and characterized (Ben-Meir et al. 2002, Winkel-Shirley 2001). To identify the potential candidate gene for *pr* in regulating the anthocyanin accumulation and investigate whether *pr* represented one of the structural genes which was significantly upregulated in the purple cauliflower mutant, we examined the expression of anthocyanin structural genes in the curd and leaf tissues of the wild type and purple cauliflower by Northern blot analysis. Cauliflower and *Arabidopsis* typically share over 85% nucleotide sequence identity in the coding regions (Babula et al. 2003, Lan et al. 2000). The *Arabidopsis* cDNAs are well suited for use as probes to detect the expression of the homolog genes in cauliflower (Li et al. 2001). The cDNAs coding *CHS*, *CHI*, *F3H*, *F3'H*, *DFR*, *LDOX*, and *UGT* in the anthocyanin biosynthetic pathway in *Arabidopsis* were used as probes for the Northern blot analysis. As shown in Figure 2.7, while the early pathway genes were expressed at similar levels between wild type and *pr* mutant in both the leaf and curd tissues, the late structural genes *BoF3'H*, *BoDFR* and *BoLDOX* were found to exhibit differential expression in both the curd and young leaf tissues. The transcript levels of these three genes were dramatically upregulated in the mutant (Figure 2.7). The co-upregulation of the three structural genes suggests that *pr* is unlikely to be the mutation of an anthocyanin structural gene. However, transcription regulators might be involved in the ectopic anthocyanin accumulation in the purple cauliflower.



**Figure 2.7.** Expression pattern of the anthocyanin biosynthetic genes in curd and leaf tissue of purple (P) and white (W) cauliflower by Northern blot analysis.

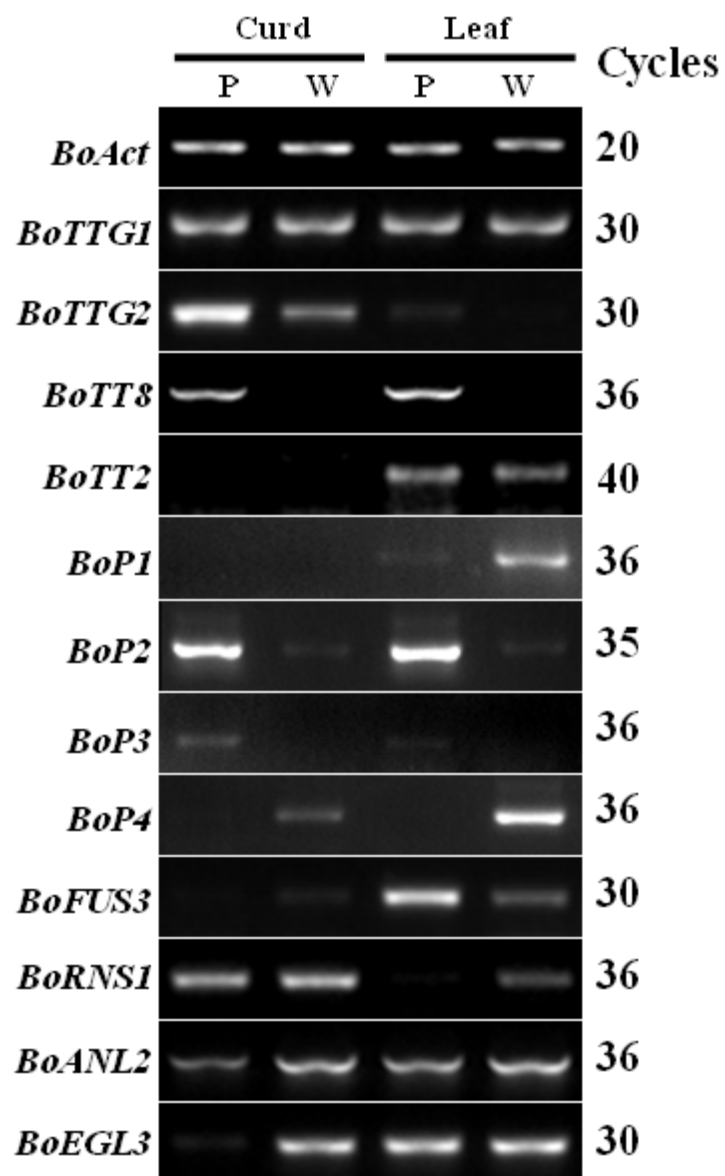


#### **2.4.6. Expression of anthocyanin regulatory genes**

Many regulatory genes that control flavonoid metabolism have been isolated (Allan et al. 2008, Dixon et al. 2005, Lepiniec et al. 2006). To investigate whether the *pr* gene represented a mutation of one of the known regulatory genes, the transcript levels of a number of flavonoid regulatory genes were examined in wild type and purple mutant plants. Since the regulatory gene families comprise of multiple paralogous genes (Cone et al. 1993, Heim et al. 2003, Ludwig and Wessler 1990, Stracke et al. 2001, Yoshida et al. 2008), the expression of a particular flavonoid regulatory gene in cauliflower was examined by semi-quantitative RT-PCR using gene-specific primers (Table A1.1). The available sequences of flavonoid regulatory genes from *Arabidopsis* and the significant amounts of *Brassica* sequence information in the public domains were used as direct resources for designing the gene-specific primers for the cauliflower homologs. In the case where a family of genes such as *MYBs* shares high sequence identities, those genes were first cloned from cauliflower in order to design gene-specific primers. Twelve regulatory genes involved in controlling flavonoid biosynthesis were examined. As shown in Figure 2.8, the mRNA levels of these regulatory genes could be detected in the curd and young leaf tissues of both genotypes. Although there were a few genes with different expression patterns between the purple and wild type samples, the *BoTT8* and the *PAP*-like (Production of Anthocyanin Pigment) MYB family genes, *BoP2* and *BoP4*, exhibited differential expression in both the leaf and curd tissues between the white and purple mutant plants.

#### **2.4.7. Tissue specific gene expression**

The anthocyanins in purple cauliflower exhibited tissue and developmental specific accumulation (Figure 2.3). To examine whether such a specific anthocyanin



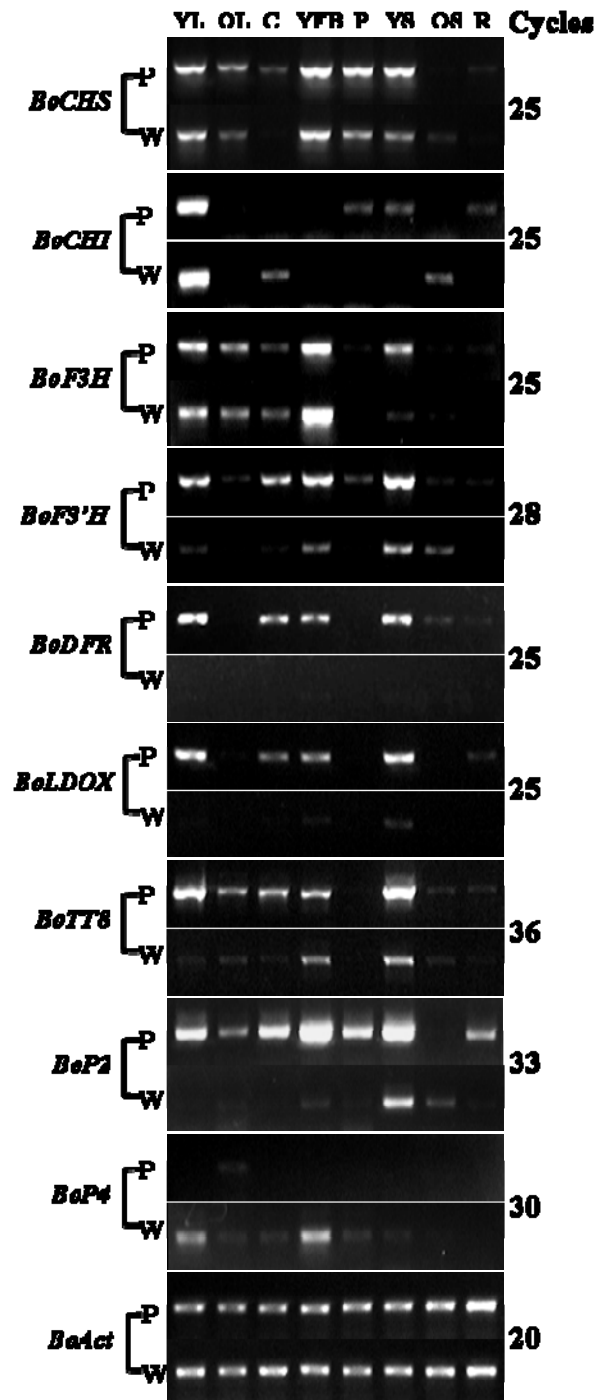
**Figure 2.8.** Expression of flavonoid regulatory genes in the curd and leaf tissue of purple (P) and white (W) cauliflower.

accumulation pattern in the mutant was correlated with the gene expression patterns, we analyzed the transcript levels of the differentially expressed structural and regulatory genes along with early structural genes in the different tissues of the *pr* mutant and wild type cauliflower by semi-quantitative RT-PCR. As shown in Figure 2.9, very similar levels of expression of early structural genes *BoCHS*, *BoCHI*, and *BoF3H* were observed in the examined tissues of the *pr* mutant and wild type plants. In contrast, the transcripts of *BoF3'H*, *BoDFR* and *BoLDOX* as well as *BoTT8* and *BoP2* were highly accumulated in very young leaves, curds, very young flower buds, and young siliques, but were absent or only present at low levels in mature leaves, flowers, old siliques, and roots in the mutant. The transcripts of these genes were at undetectable or comparably very low levels in all the tissues of WT plants. The expression of the structural genes *BoF3'H*, *BoDFR* and *BoLDOX* and that of *BoTT8* and *BoP2* were consistent with the tissue-specific anthocyanin accumulation pattern in the purple cauliflower mutant.

## 2.5. DISCUSSION

### 2.5.1. *Graffiti* represents a unique anthocyanin production mutant

Anthocyanins have been found to accumulate in several plant tissues in nature, such as roots (Mano et al. 2007), leaves (Yuan et al. 2009), fruit skin (Azuma et al. 2008, Ban et al. 2007), fruit flesh (Espley et al. 2007), petals (Park et al. 2007) and pericarp (Sweeney et al. 2006). However, only few studies on dicotyledons reported anthocyanin accumulation in the seed endosperm (Gonzalez et al. 2008). Several cauliflower varieties, including *Graffiti*, Purple Cape, Violet Queen, Violetta Italia and Rosalind, are commercially available. There also exist several varieties of red cabbage, another group of *Brassica Oleracea* vegetable, in the market (Yuan et al. 2009). *Graffiti* cauliflower is the only variety among these plants with anthocyanin



**Figure 2.9.** Gene expression pattern in tissues from different developmental stages between purple (P) and white (W) cauliflower. YL, young leaves; OL, old leaves; C, curds; YFB, young flower buds; P, petals; YS, young siliques; OS, old siliques

accumulation in the seed endosperm (Figure 2.2.J). Besides, Graffiti also exhibits young tissue specific anthocyanin accumulation, such as young siliques, young leaves, young flower buds, and curds (Figure 2.2). This unique phenotype was not seen in other plants, suggesting that in Graffiti there exists a unique tissue-specific mechanism controlling anthocyanin accumulation. Gene expression profiles for different developmental stages were consistent with tissue specific anthocyanin accumulation (Figure 2.9). However, the mechanism remains unclear. Identification and characterization of the *Purple* gene may provide more information for the mechanism underlying the tissue specific phenotype.

#### ***2.5.2. Gene expression patterns consistent with tissue specific anthocyanin accumulation in Graffiti***

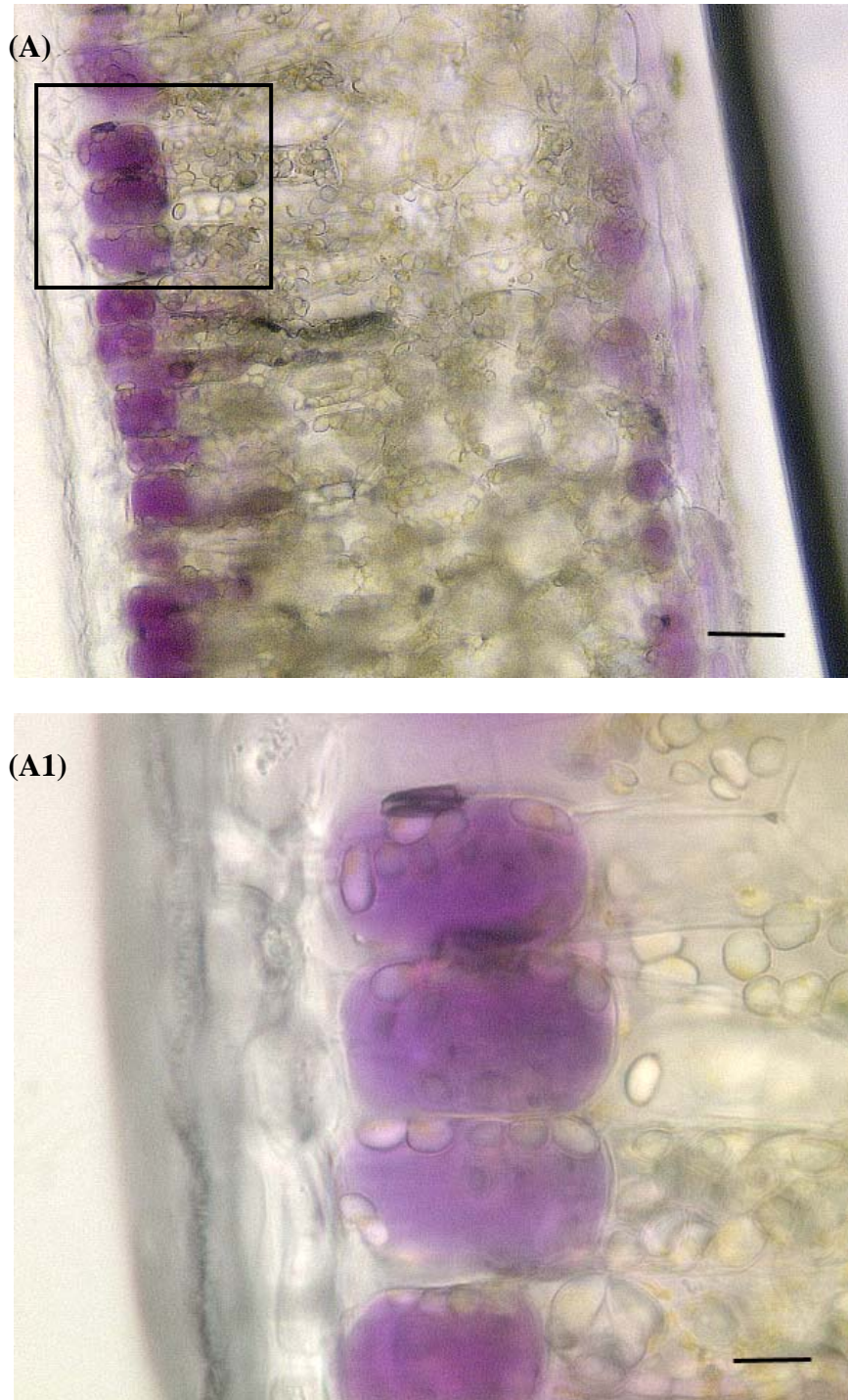
Graffiti cauliflower exhibits tissue specific anthocyanin accumulation. Gene expression analysis revealed the up-regulation of the late anthocyanin biosynthetic structural genes, *BoF3'H*, *BoDFR* and *BoLDOX*, and two anthocyanin transcription factors, *BoTT8* and *BoP2*, in the purple mutant. While the expression of *BoF3'H*, *BoDFR*, *BoLDOX* and *BoTT8* were consistent with tissue specific anthocyanin accumulation, *BoP2* seemed to be expressed in the entire mutant plants (Figure 2.9). Roots and petals of Graffiti cauliflower had no anthocyanin accumulation (Figure 2.2.A, F). Nevertheless, *BoP2* was still expressed at a relatively high level in these organs, which suggested that *BoP2* expression alone does not activate the pathway. *BoTT8* was not expressed in the roots and petals (Figure 2.9). In the old leaf tissue, although *BoTT8* and *BoP2* expressed at low levels, the late anthocyanin biosynthetic pathway structural genes did not have any expression, suggesting that the level of these proteins need to be high enough in order to activate the pathway. A recent study showing that the ratio of bHLH and MYB proteins may regulate the anthocyanin

production in orchid flower (Ma et al. 2008) supports our results. In the young siliques of wild type cauliflower, the presence of anthocyanin accumulation (Figure 2.2.G) suggested that the anthocyanin biosynthetic pathway in the wild type cauliflower remains a functional pathway. While under high light and high temperature conditions, wild type cauliflower exhibited anthocyanin accumulation (Figure 2.10), which seemed to support this theory. Examination of the gene expression patterns showed that *BoP2* and *BoTT8* are expressed at a high level (Figure 2.9) in the young siliques of wild type plants.

### **2.5.3. Abnormal *BoUGT* and *BoP4* expression pattern**

From the Northern blot results, *BoUGT*, a late anthocyanin biosynthetic pathway structural gene, exhibited higher expression in the curd tissue (Figure 2.7). Various UDP-glucose-dependent glycosyltransferases have been found in several plant species with different functions (Tanaka et al. 2008). They belong to the large glycosyltransferase family 1 (CAZy, [www.cazy.org/fam/acc\\_GT.html](http://www.cazy.org/fam/acc_GT.html)). Therefore, the expression level of *BoUGT* as detected by the Northern blot may be due to the contributions from several genes in the same family.

As in the case for *Arabidopsis*, four subgroup 6 R2R3-MYB family genes have been found in cauliflower, namely *BoP1*, *BoP2*, *BoP3*, and *BoP4*. Several orthologs have been found in other species as well, such as *MdMYB10* in apple (Chagne et al. 2007, Espley et al. 2007), *VIMYBA1* in grape (Azuma et al. 2008, Kobayashi et al. 2004), *IbMYB2* in sweet potato (Mano et al. 2007), and *AmROSEA1* in snapdragon (Schwinn et al. 2006). All of these MYB proteins serve as positive regulators of anthocyanin biosynthesis. In red cabbage, *BoMYB2*, a MYB gene in the same subgroup as *BoP2* and *BoP4*, was found to be highly expressed compared to that in green cabbage (Yuan et al. 2009). However, *BoMYB3* exhibited a reversed pattern



**Figure 2.10.** Under high light high temperature condition, wild type cauliflower exhibited anthocyanin accumulation in the sub-epidermal cells layer of old leaves. Scale bars: A, 30  $\mu\text{m}$ ; A1, 10  $\mu\text{m}$

which hinted at an interesting balance within this subgroup (Yuan et al. 2009). In cauliflower, the different expression of *BoP2* and *BoP4* was the same as the homologues in red cabbage (Figure 2.8). It is still unclear why a positive anthocyanin biosynthetic regulator such as *BoP4* is expressed to higher level in the wild type plant, but does not result in anthocyanin accumulation. Further functional studies of *BoP2* and *BoP4* protein may provide us with more information to understand their roles in regulating anthocyanin accumulation.

#### ***2.5.4. Potential candidate genes in regulating anthocyanin accumulation in Graffiti***

Our results revealed the up-regulation of the late anthocyanin biosynthetic structural genes, *BoF3'H*, *BoDFR* and *BoLDOX*, in the purple mutant. As three biosynthetic genes were simultaneously upregulated, this result suggests that the *Pr* gene is unlikely a mutation of an anthocyanin structural gene. In *Arabidopsis*, the WD40, bHLH and MYB (WBM) complex has been shown to be specifically targeted to late anthocyanin biosynthetic structural genes (Gonzalez et al. 2008). Graffiti cauliflower exhibited over-expression of two of the transcription factors involved in the WBM complex, suggesting the possible involvement of the WBM complex in this regulation. *BoP2* and *BoTT8* (Figure 2.7 and Figure 2.8) were dramatically up-regulated in the purple mutant. The F2 segregation analysis suggested that the *pr* gene was a single, semi-dominant gene that caused the ectopic phenotype. Thus, one of these transcription factors or the gene controlling the expression of these genes is a possible candidate gene for *pr*.

Both the bHLH and MYB proteins have been shown to regulate anthocyanin biosynthesis in different plant species. Over-expression of a subgroup 6 R2R3-MYB transcriptional factor can result in anthocyanin accumulation in apple (Espley et al. 2007), sweet potato (Mano et al. 2007), *Arabidopsis* (Borevitz et al. 2000, Gonzalez



et al. 2008) and maize (Cone et al. 1993, Paz-Ares et al. 1987). Similarly, modification of the bHLH transcriptional factors altered the pigment production in maize (Burr et al. 1996) and rice (Sweeney et al. 2006). Furthermore, the expression of these transcription factors can be regulated by other transcription factors. For example, the expression of *TT8* can be regulated by the bHLH proteins EGL3 and GL3, and the MYB protein, TT2 (Baudry et al. 2006, Gonzalez et al. 2008) in *Arabidopsis*. In purple cauliflower, both *MYB* and *bHLH* exhibited up-regulation, and it is likely that the purple mutation causes the activation of *BoTT8* and *BoP2*, which in turn up-regulates *F3'H*, *DFR* and *LDOX* expression, leading to anthocyanin accumulation in the purple cauliflower. Future studies through genetic analysis and candidate gene analysis will help to identify *pr* in the purple cauliflower Graffiti responsible for regulating the elevated anthocyanin production.

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## CHAPTER 3

### CANDIDATE GENE MAPPING OF THE *PURPLE* GENE THAT CONTROLS ANTHOCYANIN BIOSYNTHESIS IN THE PURPLE CAULIFLOWER MUTANT

#### 3.1. ABSTRACT

The accumulation of anthocyanins in purple cauliflower Graffiti is controlled by a semi-dominant gene, *Purple*. Here we performed candidate gene analysis in order to identify the *Purple* locus. Previously, we had identified several genes that exhibited differential expression between the wild type and the mutant, including *BoF3'H*, *BoDFR*, *BoLDOX*, *BoP2*, *BoP4*, and *BoTT8*. These differentially expressed genes as well as the genes regulating their expression, including *BoTT2*, *BoEGL3* and *BoTTG1*, were selected as the candidate genes for association analysis using RFLP and SNP markers. A R2R3-MYB transcription factor, *BoP2*, was found to co-segregate with the *Purple* locus. Sequence analysis further revealed that different transposable elements were inserted in the promoter region of the *BoP2* alleles, leading to the hypothesis that the differential expression of *BoP2* was due to differences in the promoter.

#### 3.2. INTRODUCTION

The anthocyanin biosynthetic pathway is among the most extensively studied secondary metabolic pathways, and numerous structural and regulatory genes have been cloned from several species, such as *Arabidopsis*, maize, petunia and snapdragon (Grotewold 2006, Stafford 1990). In order to identify the single semi-dominant *Purple* gene in regulating anthocyanin biosynthesis in the purple cauliflower, Graffiti, a candidate gene analysis approach was used. Cauliflower (*Brassica oleracea* L var. *botrytis*) is closely related to the model plant *Arabidopsis* with over 85% nucleotide



sequence identity in the coding regions (Babula et al. 2003, Lan et al. 2000). The functional and sequence information of the anthocyanin biosynthetic and regulatory genes available in the TAIR database ([www.arabidopsis.org](http://www.arabidopsis.org)) facilitated the cloning of the orthologous genes from cauliflower for candidate gene analysis.

The candidate gene approach is based on the concept that a genetic variation of a phenotype under investigation is the result of the mutation of specific genes (Pflieger et al. 2001). A candidate gene can be a structural gene or a regulatory gene with known functions for a metabolic pathway that directly or indirectly affects the trait of interest. The candidate gene approach has been successfully applied to fruit color research in pepper (Huh et al. 2001), indicating the feasibility of this approach in studying the anthocyanin synthesis in purple cauliflower mutant. Based on the gene expression analysis of the structural and regulatory genes of the anthocyanin biosynthetic pathway in purple cauliflower in Chapter 2, we identified the late pathway structural genes, *BoF3'H*, *BoDFR* and *BoLDOX*, the MYB transcription factors *BoP2* and *BoP4* and the bHLH transcription factor *BoTT8* as genes more highly expressed in purple cauliflower. Therefore these genes or the genes regulating their expression were selected as the prime candidates for the *Purple* gene.

Several marker systems are available nowadays. Restriction fragment length polymorphisms (RFLPs) markers are designed based on the differences in the restriction enzyme cutting sites between two individuals due to mutation (Jones et al. 2009, Tanksley et al. 1989). The advantages of this marker are its co-dominance and high reproducibility, while the disadvantage is that the availability of this marker is dependent on genetic differences between two individuals (Jones et al. 2009); in other words, if the two individuals are genetically too similar, one may not be able to find polymorphisms.

Single nucleotide polymorphisms (SNPs) are the most common variants in the genomes of eukaryotes. The frequency of SNPs may vary in different regions of a gene. For example, in maize, the average was 1 SNP per 31 bp in the noncoding regions and 1 per 124 bp in the coding regions (Tyrka et al. 2004). The advantages of the SNPs marker include its co-dominance, wide distribution across the genome, and high through. The disadvantage is that sequence information is required to design the markers.

With the availability of *Arabidopsis* sequence information, we first performed a tentative candidate gene analysis to identify the *Purple* gene using RFLP markers. Bulk DNA pools from the 8 WT and 8 *Pr* homozygous progeny derived from the F<sub>2</sub> mapping population were used to identify potential RFLP markers. However, no polymorphisms were detected for the DNA digested with 28 restriction enzymes using *BoDFR*, *BoLDOX*, *BoTT8*, *BoTT2*, *BoEGL3* and *BoTTG1* probes. The results indicated that the two parents we selected to generate the mapping population were genetically very similar to each other. Subsequently, we cloned the alleles of those candidate genes from both the purple mutant and WT plants for the design of the SNPs markers. We later determined that a R2R3-MYB transcription factor, namely *BoP2*, co-segregated with the *Purple* gene.

### **3.3. MATERIALS AND METHODS**

#### ***3.3.1. Plant materials and growth conditions***

A mapping population of over 1898 F<sub>2</sub> individuals was generated from a cross between Graffiti and Stovepipe, and then by selfing the heterozygous F<sub>1</sub> plants as described in Chapter 2. For high resolution mapping, the genotypes of the F<sub>2</sub> individuals were visually determined based on the purple pigmentation of the two days old seedlings germinated on wet filter papers under a 14-h-light/10-h-dark cycle at

24°C in the growth chamber. Seedlings with unclear phenotype will not keep growing in the soil for DNA extraction. Cauliflower F2 plants, as well as the two parent plants, were then grown in a greenhouse under a 14-h-light/10-h-dark cycle at 23°C, followed by collection of old leaves with over 40 cm<sup>2</sup> for DNA extraction.

### **3.3.2. DNA extraction**

Total genomic DNAs from the leaves of the cauliflower plants were isolated following the method as described (Dellaporta et al. 1983) with slight modifications. Cauliflower leaf tissues (100 mg) were frozen in liquid nitrogen and crushed into powder in a FastPrep instrument (Thermo Scientific, USA). The DNAs in the leaf samples were extracted in 750 µL of the DNA extraction buffer containing 100 mM Tris pH 8.0, 50 mM EDTA pH 8.0, 500 mM NaCl, 1% SDS, and fresh added 1M β-mercaptoethanol at 65°C for 10 min. The homogenates were then mixed with 150 µl 5M KOAc on ice for 15 min and centrifuged. The DNAs were subsequently precipitated with isopropanol, washed with 70% ethanol, and resuspended in 100 µl TE containing 20 µg/mL RNase.

### **3.3.3. RFLP mapping for the structural and regulatory genes**

DNA of 8 white and 8 purple cauliflowers from the F2 population were extracted individually, and each phenotypic group was then bulked together for identifying RFLP markers. The bulked genomic DNA (total 32 µg, 4 µg each) of the 8 F2 plants were digested with 28 restriction enzymes (*Apa*I, *Xho*I, *Kpn*I, *Sac*I, *Xba*I, *Spe*I, *Eco*RV, *Pvu*I, *Pst*I, *Not*I, *Xma*I, *Hinc*II, *Cla*I, *Bam*HI, *Hind*III, *Pvu*II, *Bgl*II, *Asc*I, *Mlu*I, *Hpa*I, *Bsp*HI, *Nco*I, *Dra*I, *Sac*II, *Acc*I, *Ssp*I, *Sca*I and *Eco*RI), separated on a 0.8% w/v agarose gel, and blotted onto Hybond N<sup>+</sup> membranes using the regular procedures (Sambrook and Russell 2006). The membranes were hybridized with <sup>32</sup>P-labeled

probes (Feinberg and Vogelstein 1984) overnight, and then washed twice in  $2\times$  SSC and 0.1% w/v SDS for 5 min each at  $65^{\circ}\text{C}$ , and exposed on Kodak BioMax MS film for 1–2 days.

#### **3.3.4. Genome walking**

PCR walking on the genomic DNA was performed using the Universal GenomeWalker kit following the manufacturer's instructions (Clontech, Mountain View, CA) as described previously (Li and Garvin 2003). Wild type and mutant cauliflower genomic DNAs (2.5  $\mu\text{g}$ ) were digested with *HincII*, *RsaI*, *SmaI*, *StuI*, *SwaI*, *DraI*, *EcoRV*, *PvuII*, *ScaI*, and *SspI*, respectively, and ligated to the GenomeWalker adaptors to generate the Genome walking libraries. The primers designed for genome walking were based on the known sequence of each gene at its 5' and 3' ends and had  $T_m$  values over  $65^{\circ}\text{C}$ . The PCR products from the secondary nested reactions were cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA) and sequenced.

#### **3.3.5. Sequence analysis**

DNA contigs were built using the DNASTar program (Lasergene, Madison, WI) and multiple sequence analysis was performed by Vector NTI (Invitrogen, Carlsbad, CA) or DNASTar using the default settings. The phylogenic tree was calculated by DNASTar and visualized by Treeview version 1.6.6. ([taxonomy.zoology.gla.ac.uk/rod/treeview.html](http://taxonomy.zoology.gla.ac.uk/rod/treeview.html)).

#### **3.3.6. Design of ARMS-PCR markers for associative analysis**

DNA was extracted from the parents and 102 F2 individuals of the mapping population as described in Chapter 2. For the design of the PCR-based markers, both alleles of the candidate genes from the purple mutant and WT plants were isolated and

sequenced. Based on single nucleotide polymorphisms in the two alleles of these genes, primer sets *BoEGL3* (85, 86, 87 and 88) and *BoTT2* (89, 90, 91 and 92) were used for the tetra-primer ARMS-PCR analysis (Table A.1.1) (Chiapparino et al. 2004), with both sets being co-dominant markers. Inner primers with one single nucleotide difference at the 3' end were used to distinguish the single nucleotide polymorphism (Figure 3.1). Primers with  $T_m$  over  $57^{\circ}\text{C}$  were essential for this marker system and the  $T_m$  difference within each primer set should not exceed  $2^{\circ}\text{C}$  to prevent the amplification of non-specific alleles. The PCR profiles were designated to be 10 cycles for the lowest  $T_m$  of the primer set as the annealing temperature, and then 25 cycles for the lowest  $T_m$  minus  $3^{\circ}\text{C}$  as the annealing temperature.

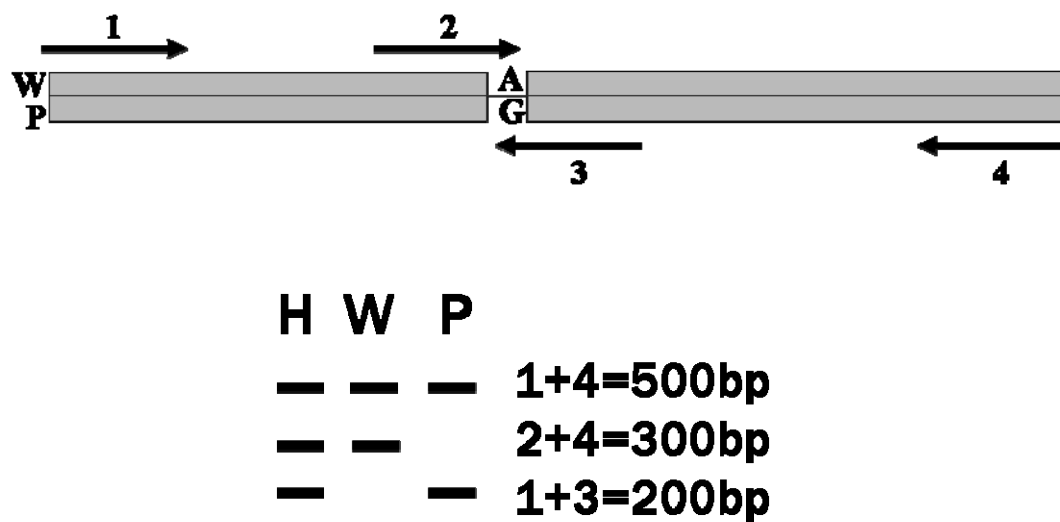
### ***3.3.7. Associative mapping of the PAP-like genes***

DNAs of the parents and 1898 F2 individuals of the mapping population were extracted. Both alleles of the *PAP*-like genes from the purple mutant and WT plants were isolated and sequenced. Based on the insertions and deletions in the two alleles of these genes, primers sets *BoP2* (57, 58 and 59), *BoP3* (60 and 61) and *BoP4* (62, 63 and 64) were used for mapping. The *BoP2* and *BoP3* were co-dominant markers (Figure 3.2.A), whereas the *BoP3* was a dominant marker (Figure 3.2.B). Primers with  $T_m$  over  $57^{\circ}\text{C}$  were essential to prevent the amplification of other similar sequences of the MYB genes. The mapping data and graph were analyzed and generated using MapMaker ([www.broadinstitute.org/ftp/distribution/software/mapmaker3](http://www.broadinstitute.org/ftp/distribution/software/mapmaker3)).

## **3.4. RESULTS**

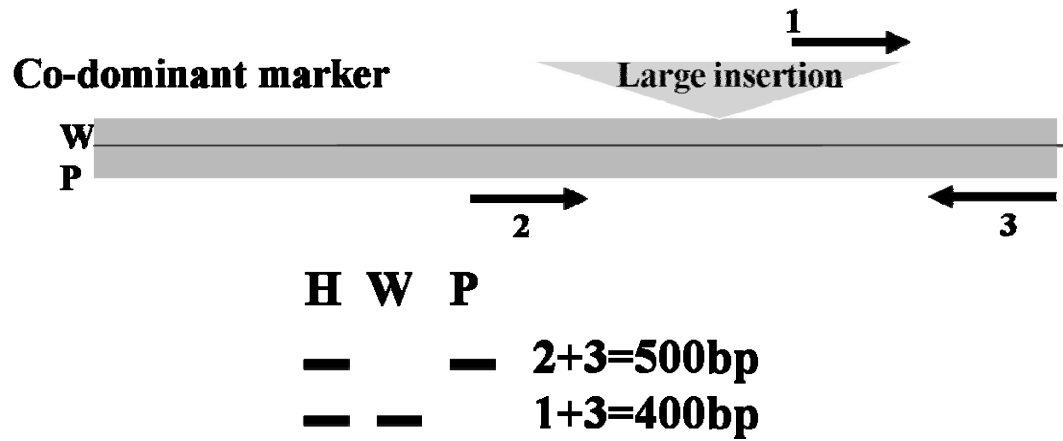
### ***3.4.1. RFLP mapping of the candidate genes***

According to the gene expression profiles (Figure 2.7 and Figure 2.8) for the purple cauliflower Graffiti in Chapter 2, we hypothesized that the possible candidate genes

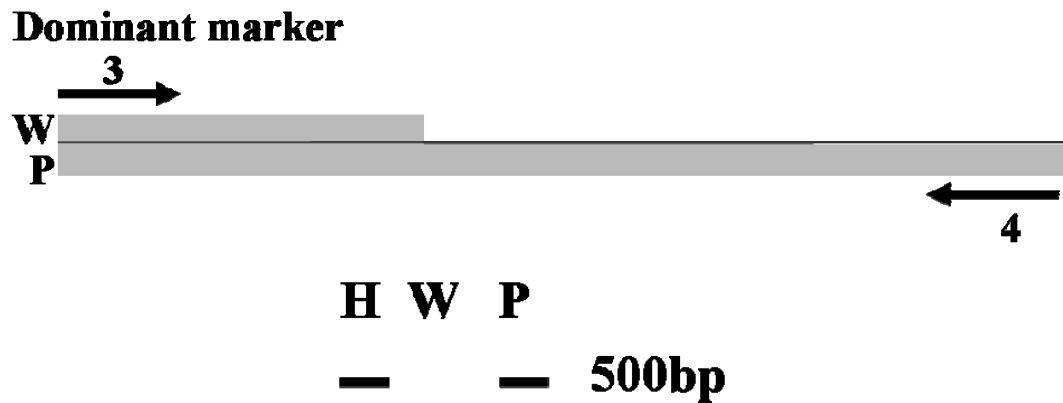


**Figure 3.1.** An example of tetra-primer ARMS-PCR marker design. P: homozygous mutant, W: homozygous wild type, H: heterozygous

(A)



(B)



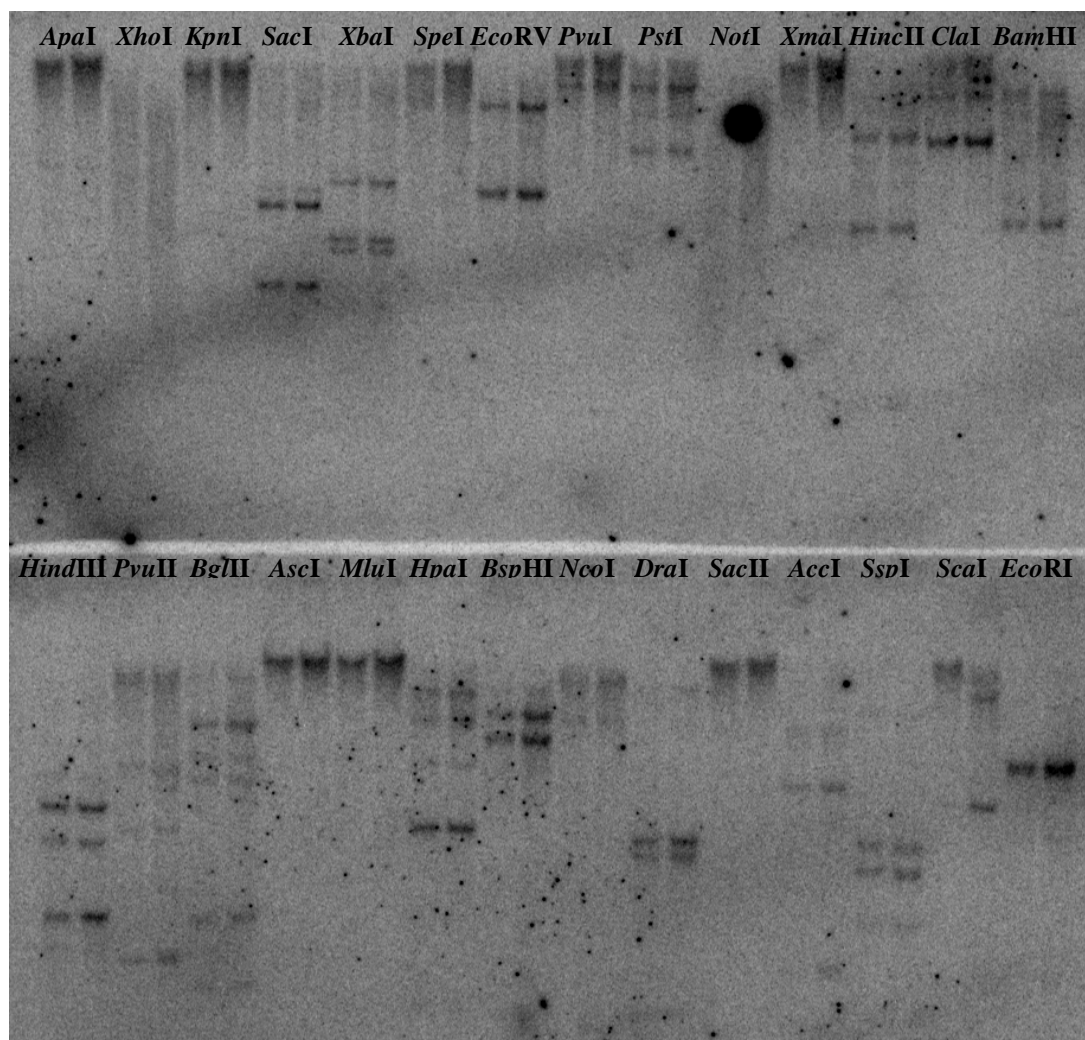
**Figure 3.2.** Examples of markers designed based on insertion/deletion in the gene. (A) Co-dominant marker, (B) Dominant marker, P: homozygous mutant, W: homozygous wild type, H: heterozygous

for *pr* are likely the genes exhibiting up-regulation in Graffiti, which included the structural genes *BoF3'H*, *BoDFR* and *BoLDOX*, and the regulatory genes *BoTT8* and *BoP2*, or the genes controlling the expression of these genes, such as *BoTT2*, *BoEGL3*, *BoTTG1*. Although the *pr* gene is unlikely to represent an allele of the *BoF3'H*, *BoDFR* or *BoLDOX* gene, RFLP mapping to examine the association of *pr* with these three biosynthetic genes along with the regulatory genes was nevertheless attempted. Cauliflower and *Arabidopsis* share over 85% nucleotide sequence identity in the coding regions (Babula et al. 2003, Lan et al. 2000), thus enabling us to use known gene fragments of *Arabidopsis* as probes for the cauliflower orthologs with unknown sequence. We first examined *BoDFR*, *BoLDOX*, *BoTT8*, *BoTT2*, *BoEGL3* and *BoTTG1* as they are single copy gene sequences in *Arabidopsis* (Winkel-Shirley 2001). To develop the RFLP markers, the bulked DNAs were digested with a total of 28 restriction enzymes and hybridized with those gene probes. However, none of them exhibited polymorphisms between the wild type and the mutant (Figure 3.3 and Figure 3.4). This result suggested that the two parental cultivars may be genetically too similar to each other, to the extent that we were unable to detect any polymorphisms for the development of RFLP markers. Therefore, we decided to clone and sequence the candidate genes in order to design specific markers based on their sequence polymorphisms.

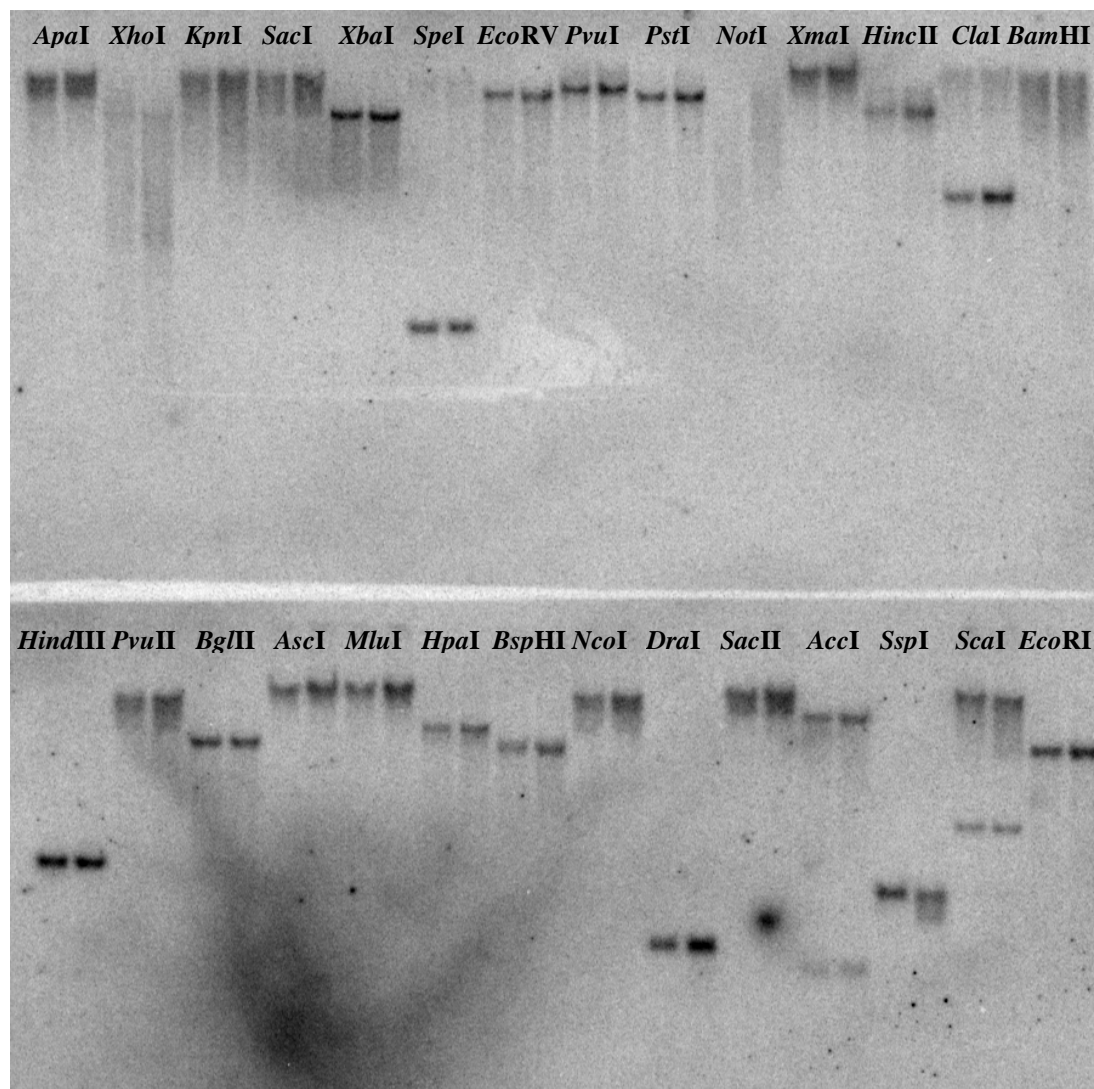
#### **3.4.2. Sequence analysis of regulatory genes**

Since a set of late anthocyanin biosynthetic pathway genes were turned on in the purple cauliflower Graffiti (Figure 2.7), we hypothesized that the *Purple* gene is likely to be a transcription factor. We amplified several transcription factors using the same set of primers as were used for the RT-PCR analysis in Chapter 2 to obtain the sequence information from both wild type and mutant plants. Genome walking was





**Figure 3.3.** Developing RFLP markers with the use of *LDOX* as a probe. DNAs of the mutant (left lane) and the wild type (right lane) cauliflower were digested with different restriction enzymes and then hybridized with *LDOX* probe.

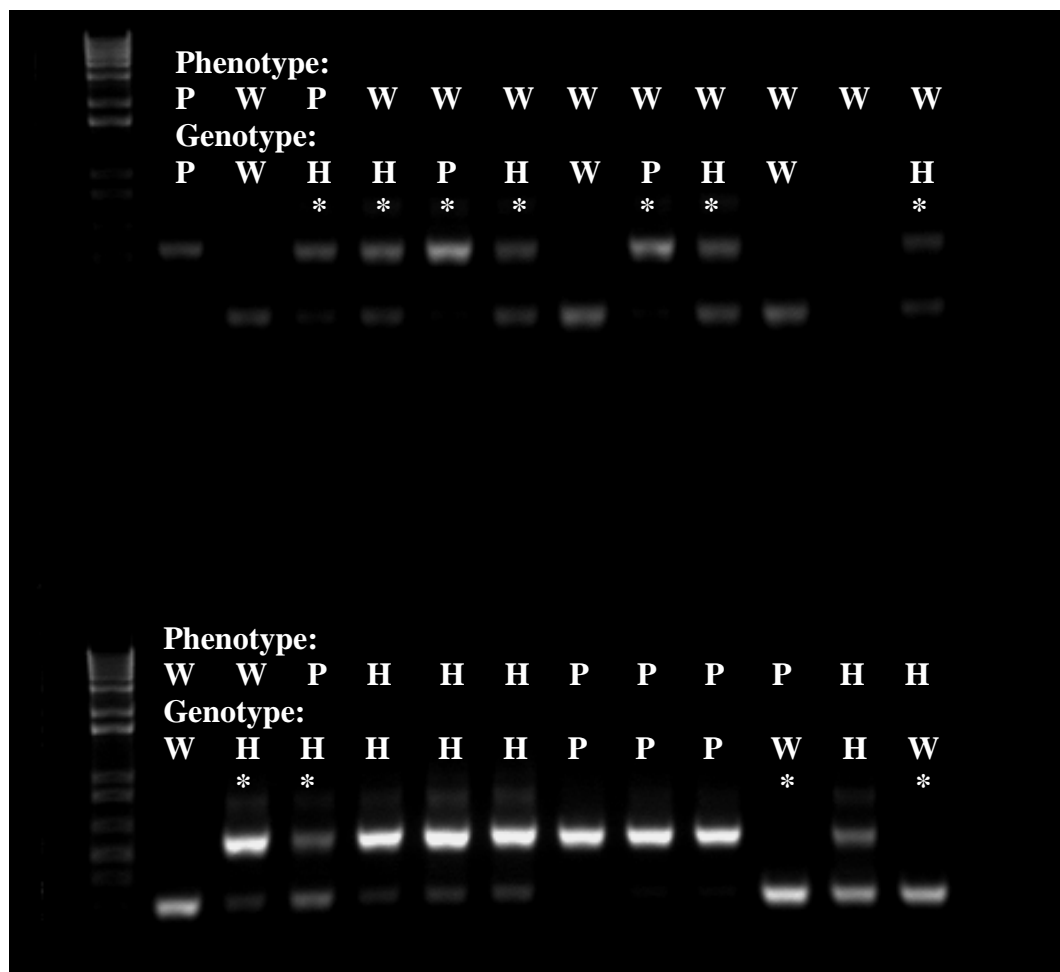


**Figure 3.4.** Developing RFLP markers with the use of *TT2* as a probe. DNAs of the mutant (left lane) and the wild type (right lane) cauliflower were digested with different restriction enzymes and then hybridized with *TT2* probe.

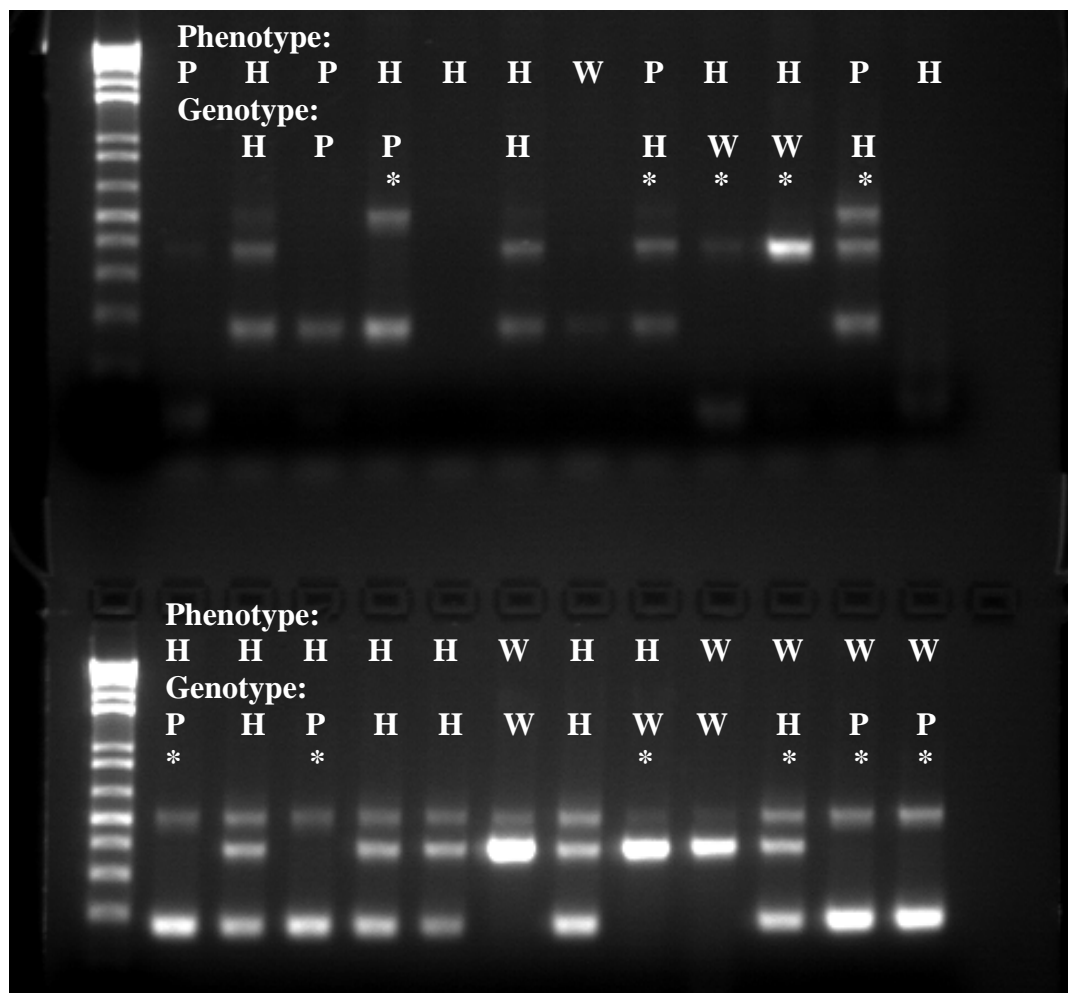
performed to acquire the sequence information outside of these regions. Sequence information for *BoTT8*, *BoP2* and *BoP4*, which exhibited differential expression between the two parents (Figure 2.8), as well as *BoP1*, *BoP3*, *BoTTG1*, *BoTT2* and *BoEGL3*, whose orthologs in *Arabidopsis* demonstrated an ability to regulate the expression of *TT8* (Baudry et al. 2006, Gonzalez et al. 2008), was obtained. No sequence polymorphisms were found between the mutant and wild type alleles for *BoTT8*, *BoP1* and *BoTTG1* (Figure 2.8). Several single nucleotide polymorphisms and insertions/deletions were found in *BoTT2*, *BoEGL3*, *BoP2*, *BoP3* and *BoP4*. Thus, we used this information in designing the PCR-based gene specific markers for association analysis.

#### ***3.4.3. BoTT2 and BoEGL3 were ruled out as candidates for the Purple gene***

In *Arabidopsis*, PAP1 and TT8 are known to specifically activate the expression of *F3'H*, *DFR*, and other late pathway genes in regulating the anthocyanin biosynthesis (Baudry et al. 2006, Gonzalez et al. 2008, Nesi et al. 2000). Thus, the differentially expressed genes *BoTT8* and *BoP2*, or the genes that regulate their expression, are likely the candidate genes for *pr*. Although the expression pattern of *BoTT2* and *BoEGL3* seemed non-specific (Figure 2.8), previous studies had shown that they were capable of regulating the expression of *BoTT8* (Baudry et al. 2006). We therefore first mapped these two genes using the tetra-primer ARMS-PCR markers (Chiapparino et al. 2004) that were designed based on the single nucleotide polymorphisms between the mutant and the wild type alleles (Figure 3.1). We then screened the 102 F2 population described in Chapter 2; 11 out of 20 and 51 out of 79 recombinant plants for the *BoEGL3* and *BoTT2*, respectively, were found within the population (Figure 3.5 and Figure 3.6), suggesting that neither *BoEGL3* nor *BoTT2* was the *Purple* gene.



**Figure 3.5.** An example of mapping for *BoEGL3*. P: homozygous mutant, W: homozygous wild type, H: heterozygous, \* represent recombinant plants

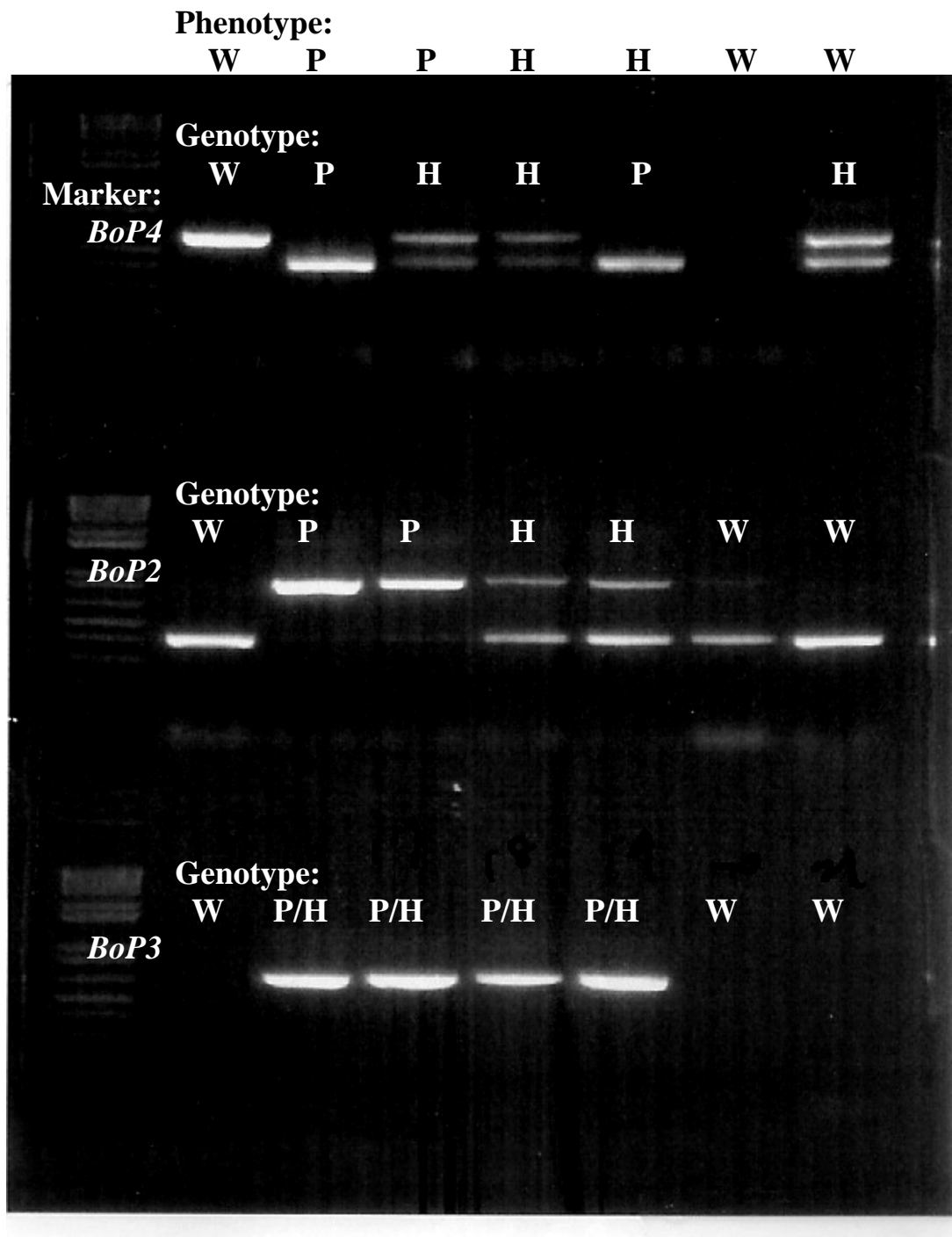


**Figure 3.6.** An example of mapping for *BoTT2*. P: homozygous mutant, W: homozygous wild type, H: heterozygous, \* represent recombinant plants

#### **3.4.4. Identification of a MYB gene that co-segregated with *pr***

*Arabidopsis* PAP1, together with TT8, EGL3 or GL3, can form a complex to regulate the expression of *TT8*. While *BoP1-P4* and *BoTT8* exhibited differential expression patterns between the mutant and the wild type plants, *BoTT8* and *BoP1* shared no sequence polymorphisms between the two alleles. Besides, other *TT8* regulators such as *BoTT2* and *BoEGL3* were not linked to the *Purple* gene. Taken together, one of the *PAP*-like genes, *BoP2*, *BoP3* and *BoP4*, is likely to be the *Purple* gene. Based on different insertion/deletions between the two alleles, PCR based markers for the *PAP*-like genes were developed. Three of the *PAP*-like genes, *BoP2*, *BoP3* and *BoP4*, were found to co-segregate with the *pr* locus in a mapping population of the 102 F2 plants.

The *PAP*-like genes belong to the R2R3-MYB family subgroup 6 in *Arabidopsis* (Stracke et al. 2001). This subgroup consists of 4 members, *AtMYB75* (*PAP1*), *AtMYB90* (*PAP2*), *AtMYB113* and *AtMYB114*, with three of them clustered together on chromosome one (TAIR, [www.arabidopsis.org](http://www.arabidopsis.org)). The cauliflower genome shares microsynteny with the *Arabidopsis* genome (Paterson et al. 2001, Town et al. 2006). Based on the mapping results, it is likely that *BoP2*, *BoP3* and *BoP4* are located next to one another as is the case in *Arabidopsis*. To define the gene that represents the *pr* gene, the three *PAP*-like genes were mapped in a large mapping population of 1898 F2 individuals (Figure 3.7). Two and seven recombinant events were detected for the *BoP3* and *BoP4* markers, respectively. In contrast, no recombinant events were observed for the *BoP2* markers, indicating that *BoP2* co-segregated with *pr* and was most likely to be the *pr* gene. A high resolution genetic map is shown in Figure 3.8.



**Figure 3.7.** An example of mapping for *BoP2*, *BoP3* and *BoP4*. P: homozygous mutant, W: homozygous wild type, H: heterozygous

# Kosambi Mapping Function

Segment Break Dist  $\geq 999.9$  cM

Segment Break Frac  $\geq 50.0$  %

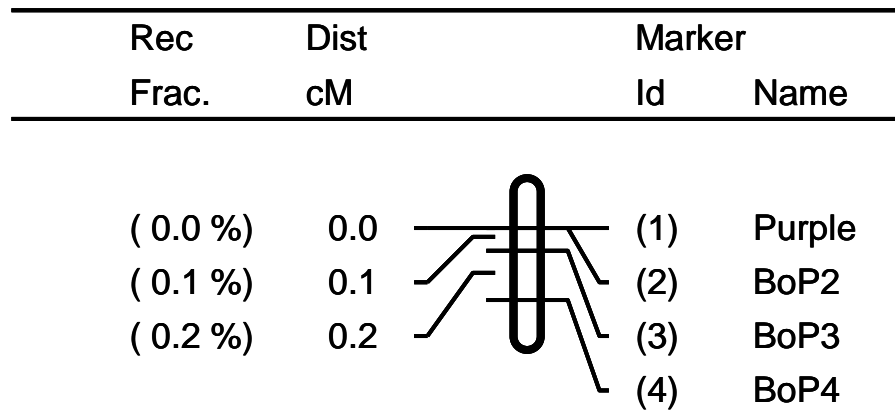
Log-Likelihood : -522.52

Iterations : 5

Longest Seg cM : 0.293

Loop Tolerance : 0.010

Inner Tolerance: 0.010



**Figure 3.8.** Linkage map of *BoP2*, *BoP3*, *BoP4* and *Purple* locus. The average ratio of genetic to physical distance is approximately 350 kb/cM in cauliflower genome.



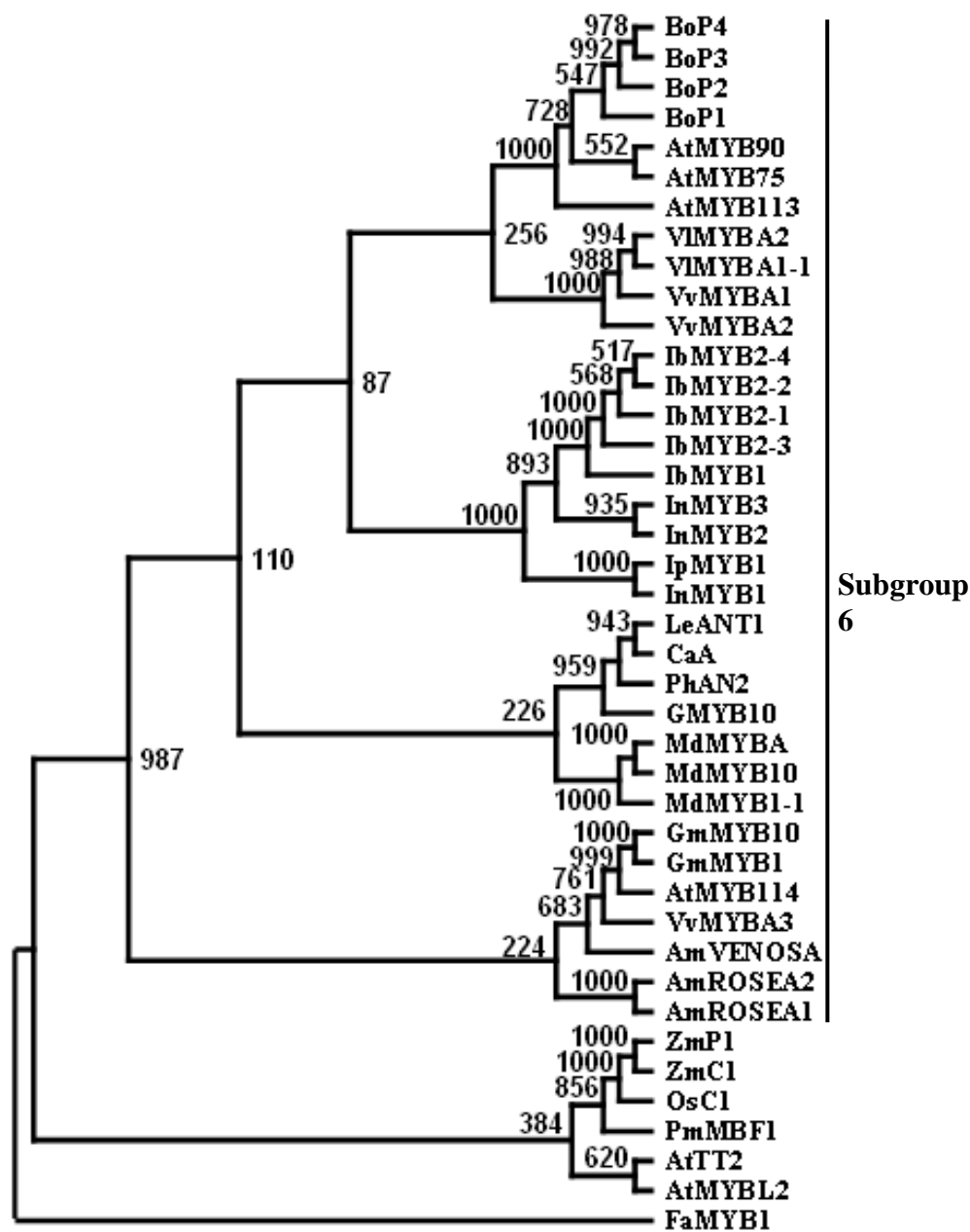
#### 3.4.5. Structural analysis of *BoP2* in cauliflower

The *BoP2* gene from both the wild type and mutant plants contains an open reading frame of 744 bp which encodes a protein of 247 amino acids with an estimated molecular mass of 27.99 kD. As with all other *PAP*-like genes in plants, the *BoP2* encodes a protein that is predicted to be a nuclear protein (WoLF PSORT, [wolfpsort.org](http://wolfpsort.org)). *BoP2* contains R2 and R3 MYB repeat domains and the conserved KPRPR[S/T]F motif which is specific to the *Arabidopsis* subgroup 6 R2R3-MYB protein (Stracke et al. 2001) (Figure 3.9). It shares approximately 79.8%-89.9% amino acid sequence homology with the other BoP proteins. Phylogenetic analysis has indicated that *BoP2* is related most closely to the *Arabidopsis* PAP proteins and belongs to the subgroup 6 of R2R3 MYB proteins involved in regulating the anthocyanin biosynthesis in other plants (Figure 3.10).

Alignment of the coding sequences of the wild type and mutant *BoP2* showed that there existed only two single nucleotide differences, which resulted in two amino acid changes from Ile to Thr and Pro to Ala at positions 14 and 159, respectively (Figure 3.9). Analysis of the promoter sequences between the two *BoP2* alleles revealed a L1 type non-LTR retrotransposon insertion at the -378 position in the wild type allele, and an autonomous DNA transposon insertion at the -373 position for the *pr* mutant allele. The transposon inserted in the promoter of *pr* encoded two proteins, one of which was a putative DNA binding protein that includes the SANT/myb/trihelix motif, and the other a 484-aa Harbinger-like DNA transposase (CENSOR, [www.girinst.org/censor/index.php](http://www.girinst.org/censor/index.php)).

	1	10	20	30	40	50	60	70	85
BoP1 WT	(1)	MEDSSKGLTKGAWTAEDSLLRRCIDKYGEKWHQVPLRAGLNRCRKSCLRWLNLYLKPS	IKRGGKLS	SDEVDLLRLRHKLGNRW					
BoP2 WT	(1)	MEGMSKGLTKGAWIAEEDNLLRQCIDKYGEKWHQVPLRAGLNRCRKSCLRWLNLYLKPS	IKRGGKLS	SDEVDLLRLRHKLGNRW					
BoP2 PR	(1)	MEGMSKGLTKGAWIAEEDNLLRQCIDKYGEKWHQVPLRAGLNRCRKSCLRWLNLYLKPS	IKRGGKLS	SDEVDLLRLRHKLGNRW					
BoP3 WT	(1)	MEGSSKRLRKGAIAEEDNLLRQCIDKYGEKWHQVPLRAGLNRCRKSCLRWLNLYLKPS	IKRGGKLS	SDEVDLLRLRHKLGNRW					
BoP4 WT	(1)	MEGSSKRLRKGAIAEEDNLLRQCIDKYGEKWHQVPLRAGLNRCRKSCLRWLNLYLKPS	IKRGGKLS	SDEVDLLRLRHKLGNRW					
At MYB75	(1)	MEGSSKGLRKGAWTTEEDSLLRQCINIKYGEKWHQVPLRAGLNRCRKSCLRWLNLYLKPS	IKRGGKLS	SDEVDLLRLRHKLGNRW					
At MYB90	(1)	MEGSSKGLRKGAIAEEDSLLRQCIDKYGEKWHQVPLRAGLNRCRKSCLRWLNLYLKPS	IKRGGKLS	SDEVDLLRLRHKLGNRW					
At MYB113	(1)	MEGSSKGLRKGAWTTEEDSLLRQCIDKYGEKWHQVPLRAGLNRCRKSCLRWLNLYLKPS	IKRGGKLS	SDEVDLLRLRHKLGNRW					
At MYB114	(1)	MEGSSKGLRKGAIAEEDSLLRQCIDKYGEKWHQVPLRAGLNRCRKSCLRWLNLYLKPS	IKRGGKLS	SDEVDLLRLRHKLGNRW					
Consensus	(1)	MEGSSKGLRKGAIAEEDSLLRQCIDKYGEKWHQVPLRAGLNRCRKSCLRWLNLYLKPS	IKRGGKLS	SDEVDLLRLRHKLGNRW					
BoP1 WT	(86)	SLIAGRLPGRRTANDIKNYWNTHLSKIH	HEP-CCKTKMKRNI	TTS	TTT	PAQ	IDV	EKPRPR	LT
BoP2 WT	(86)	SLIAGRLPGRRTANDIKNYWNTHLSKIH	HEP-GCKTQMKKRNI	PCS	SYT	TT	PAQ	IDV	EKPRPR
BoP2 PR	(86)	SLIAGRLPGRRTANDIKNYWNTHLSKIH	HEP-GCKTQMKKRNI	PCS	SYT	TT	PAQ	IDV	EKPRPR
BoP3 WT	(86)	SLIAGRLPGRRTANDIKNYWNTHLSKIH	HEP-GCKTQMKKRNI	PCS	SYT	TT	PAQ	IDV	EKPRPR
BoP4 WT	(86)	SLIAGRLPGRRTANDIKNYWNTHLSKIH	HEP-GCKTQMKKRNI	PCS	SYT	TT	PAQ	IDV	EKPRPR
At MYB75	(86)	SLIAGRLPGRRTANDIKNYWNTHLSKIH	HEP-GCKTQMKKRNI	PCS	SYT	TT	PAQ	IDV	EKPRPR
At MYB90	(86)	SLIAGRLPGRRTANDIKNYWNTHLSKIH	HEP-GCKTQMKKRNI	PCS	SYT	TT	PAQ	IDV	EKPRPR
At MYB113	(86)	SLIAGRLPGRRTANDIKNYWNTHLSKIH	HEP-GCKTQMKKRNI	PCS	SYT	TT	PAQ	IDV	EKPRPR
At MYB114	(86)	SLIAGRLPGRRTANDIKNYWNTHLSKIH	HEP-GCKTQMKKRNI	PCS	SYT	TT	PAQ	IDV	EKPRPR
Consensus	(86)	SLIAGRLPGRRTANDIKNYWNTHLSKIH	HEP-CCKTKMKRNI	TTS	TTT	PAQ	IDV	EKPRPR	LT
BoP1 WT	(171)	LNNINNV	CENSM	TYCNK	AGEK	YELF-SN	LMDG	ENM	WESL
BoP2 WT	(170)	LNDTNV	SEN	IT	IT-CNK	DDK	FELV-SN	LMDG	QNR
BoP2 PR	(170)	LNDTNV	SEN	IT	IT-CNK	DDK	FELV-SN	LMDG	QNR
BoP3 WT	(108)	LNDTNV	SEN	IT	IT-CNK	DDK	FELV-SN	LMDG	QNR
BoP4 WT	(170)	FNDANN	IGENS	FT	IT-CNK	DDK	SELV-SN	LAV	QNM
At MYB75	(170)	LN-INNV	CDNS	IT	IT-YNK	DDK	QV-LV	NN	LDG
At MYB90	(171)	LKK-NNV	CENS	IT	IT-CNK	DDK	DFV-LV	NN	LDG
At MYB113	(171)	LNN-NV	CESS	IT	IT-CNK	DE	QDKL	IN	LLD
At MYB114	(140)	LNN-NV	CESS	IT	IT-CNK	DE	QDKL	IN	LLD
Consensus	(171)	LN	NNV	ENS	IT	CNK	DDK	ELV	NLMDG
BoP1 WT	(170)	LNNINNV	CENSM	TYCNK	AGEK	YELF-SN	LMDG	ENM	WESL
BoP2 WT	(170)	LNDTNV	SEN	IT	IT-CNK	DDK	FELV-SN	LMDG	QNR
BoP2 PR	(170)	LNDTNV	SEN	IT	IT-CNK	DDK	FELV-SN	LMDG	QNR
BoP3 WT	(108)	LNDTNV	SEN	IT	IT-CNK	DDK	FELV-SN	LMDG	QNR
BoP4 WT	(170)	FNDANN	IGENS	FT	IT-CNK	DDK	SELV-SN	LAV	QNM
At MYB75	(170)	LN-INNV	CDNS	IT	IT-YNK	DDK	QV-LV	NN	LDG
At MYB90	(171)	LKK-NNV	CENS	IT	IT-CNK	DDK	DFV-LV	NN	LDG
At MYB113	(171)	LNN-NV	CESS	IT	IT-CNK	DE	QDKL	IN	LLD
At MYB114	(140)	LNN-NV	CESS	IT	IT-CNK	DE	QDKL	IN	LLD
Consensus	(171)	LN	NNV	ENS	IT	CNK	DDK	ELV	NLMDG
BoP1 WT	(170)	LNNINNV	CENSM	TYCNK	AGEK	YELF-SN	LMDG	ENM	WESL
BoP2 WT	(170)	LNDTNV	SEN	IT	IT-CNK	DDK	FELV-SN	LMDG	QNR
BoP2 PR	(170)	LNDTNV	SEN	IT	IT-CNK	DDK	FELV-SN	LMDG	QNR
BoP3 WT	(108)	LNDTNV	SEN	IT	IT-CNK	DDK	FELV-SN	LMDG	QNR
BoP4 WT	(170)	FNDANN	IGENS	FT	IT-CNK	DDK	SELV-SN	LAV	QNM
At MYB75	(170)	LN-INNV	CDNS	IT	IT-YNK	DDK	QV-LV	NN	LDG
At MYB90	(171)	LKK-NNV	CENS	IT	IT-CNK	DDK	DFV-LV	NN	LDG
At MYB113	(171)	LNN-NV	CESS	IT	IT-CNK	DE	QDKL	IN	LLD
At MYB114	(140)	LNN-NV	CESS	IT	IT-CNK	DE	QDKL	IN	LLD
Consensus	(171)	LN	NNV	ENS	IT	CNK	DDK	ELV	NLMDG
BoP1 WT	(170)	LNNINNV	CENSM	TYCNK	AGEK	YELF-SN	LMDG	ENM	WESL
BoP2 WT	(170)	LNDTNV	SEN	IT	IT-CNK	DDK	FELV-SN	LMDG	QNR
BoP2 PR	(170)	LNDTNV	SEN	IT	IT-CNK	DDK	FELV-SN	LMDG	QNR
BoP3 WT	(108)	LNDTNV	SEN	IT	IT-CNK	DDK	FELV-SN	LMDG	QNR
BoP4 WT	(170)	FNDANN	IGENS	FT	IT-CNK	DDK	SELV-SN	LAV	QNM
At MYB75	(170)	LN-INNV	CDNS	IT	IT-YNK	DDK	QV-LV	NN	LDG
At MYB90	(171)	LKK-NNV	CENS	IT	IT-CNK	DDK	DFV-LV	NN	LDG
At MYB113	(171)	LNN-NV	CESS	IT	IT-CNK	DE	QDKL	IN	LLD
At MYB114	(140)	LNN-NV	CESS	IT	IT-CNK	DE	QDKL	IN	LLD
Consensus	(171)	LN	NNV	ENS	IT	CNK	DDK	ELV	NLMDG

**Figure 3.9.** Sequence alignment of cauliflower and *Arabidopsis* subgroup 6 R2R3-MYB proteins. Two single nucleotide difference resulted in two amino acid changes from Ile to Thr and Pro to Ala at positions 14 and 159 (arrow), respectively.



**Figure 3.10** Phylogenetic tree of MYB transcription factors responsible for anthocyanin biosynthesis from different plant species. Numbers along branches indicate the percentage of bootstrap support and the values were determined from 1000 trials.

### 3.5. DISCUSSION

#### 3.5.1 *High resolution mapping revealed BoP2 to be the Purple gene*

According to the gene expression profiling, we selected *BoF3'H*, *BoDFR*, *BoLDOX*, *BoP1*, *BoP2*, *BoP3*, *BoP4*, *BoTT8*, *BoEGL3*, *BoTT2* and *BoTTG1* as the candidate genes. Using RFLPs markers and SNPs markers, we found that *BoP2*, *BoP3* and *BoP4* all co-segregated with the *Purple* locus using 102 F2 included as a mapping population. Through sequence analysis, we found *BoP2*, *BoP3* and *BoP4* to be orthologs of the *Arabidopsis* subgroup 6 R2R3-MYB genes (Figure 3.10), and all are positive regulators of the anthocyanin biosynthesis (Stracke et al. 2001). In order to determine the gene encoded within the *Purple* locus, high resolution mapping with an additional 1796 F2 plants was performed to screen for additional recombinant plants. Our mapping results revealed that *BoP3* and *BoP4* were 0.1 and 0.3 cM away from the *Purple* locus respectively (Figure 3.8). The average ratio of genetic to physical distance in cauliflower is approximately 350 kb/cM (Li et al. 2003). Hence the physical distances of *BoP3* and *BoP4* to the *Purple* locus were estimated to be 35 kb and 105 kb, respectively. Only *BoP2* co-segregated with the *Purple* locus, suggesting that the *Purple* locus most likely is *BoP2*, which regulates anthocyanin biosynthesis. In *Arabidopsis* (TAIR, [www.arabidopsis.org](http://www.arabidopsis.org)) and grape (Walker et al. 2007), the subgroup 6 R2R3-MYB genes are located in tandem. We therefore did not exclude the possibility that the *Purple* locus encoded the gene next to *BoP2*. Phenotypic complementation by transforming the *BoP2* mutant allele into wild type cauliflower is needed to confirm whether *BoP2* is indeed the *Purple* gene.

#### 3.5.2. *Total of four PAP-like genes found in cauliflower*

A total of four different *PAP*-like genes, *BoP1*, *BoP2*, *BoP3* and *BoP4* had been identified and cloned from the wild type and mutant cauliflower. All the *PAP*-like

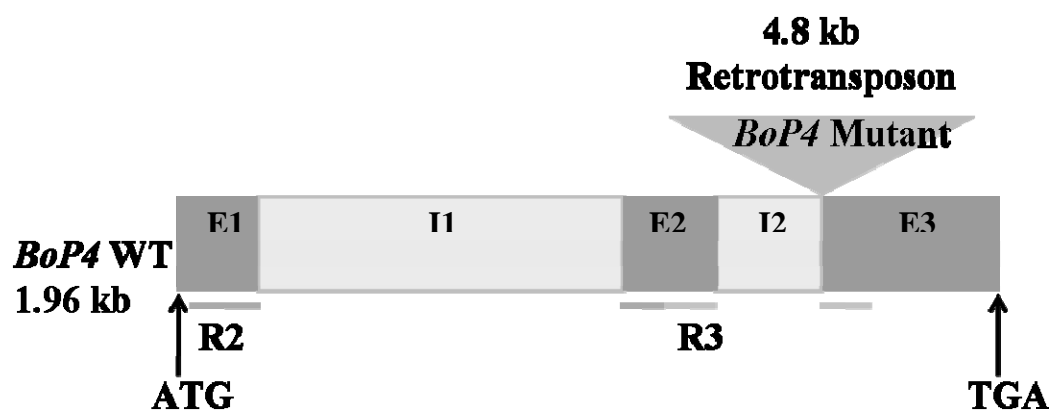
genes contain 3 exons, a long variant intron 1 and a short intron 2. All of them contain the conserved KPRPR[S/T]F motif (Figure 3.9) except for BoP1, which has L at the [S/T] position. BoP1 is less similar to other PAP-like proteins, with the sequence 77%-79.8% identical to other PAP-like proteins. No sequence differences have been found between the mutant and wild type alleles in their introns and exons.

*BoP3* includes a stop codon at the 107<sup>th</sup> amino acid located in the 3<sup>rd</sup> exon and therefore encodes a truncated protein (Figure 3.9). A previous study suggested that the minimal domain in a MYB transcription factor that a R/B-like bHLH protein can recognize is between the 53<sup>th</sup>-114<sup>th</sup> amino acids located in the R3 region (Zimmermann et al. 2004). Therefore, the stop codon located at the 107<sup>th</sup> position may result in a loss of BoP3 function.

The *BoP4* mutant allele contains a retrotransposon insertion between the junction of the 2<sup>nd</sup> intron and 3<sup>rd</sup> exon in comparison with wild type allele (Figure 3.11). This retrotransposon insertion may result in the low transcript abundance of *BoP4* in the mutant plant (Figure 2.8). All of the four *PAP*-like genes in *Arabidopsis* function as positive regulators of anthocyanin synthesis; over-expression of any of these genes results in increased pigment production (Borevitz et al. 2000, Gonzalez et al. 2008), suggesting that the R2R3-MYB genes in this subgroup are the activators of the anthocyanin biosynthetic pathway. Therefore, it is likely that BoP4 is an activator. However, it is still unclear why *BoP4* is expressed higher in the wild type cauliflower (Figure 2.8) but does not result in anthocyanin accumulation (Figure 2.2).

### ***3.5.3. The two alleles of BoP2 have retrotransposon/ transposon insertion at the -378/-373 position of their promoters.***

Sequence analysis of the promoter regions of both *BoP2* alleles revealed different transposable element insertions at the -378/-373 position, suggesting that this position



**Figure 3.11.** The *BoP4* mutant allele contains a retrotransposon insertion between the junction of the 2<sup>nd</sup> intron and 3<sup>rd</sup> exon in comparison with wild type allele. E: exon, I: intron

may be a hot spot for transposable element recognition. However, we were unable to obtain the full transposable element sequence information due to the difficulty in sequencing the entire transposable element, and therefore we were not able to identify the transposable element recognizing sequence.

Comparison of the sequences between the wild type and the mutant alleles of *BoP2* revealed that sequence polymorphisms were located in the promoter. The differential expression of *BoP2* was likely due to the differences in promoter sequence. Transposable elements can play an important role in gene regulation in plants (Feschotte et al. 2002). Retrotransposon insertion at the promoter of grape *VvmybA1* (Kobayashi et al. 2004), and transposon insertion at the second intron of maize *R* (Walker et al. 1997) resulted in mutation of the gene and loss of pigmentation. On the contrary, transposon insertion in the promoter region of a gene can also increase gene expression (Scordilis et al. 1987). The cauliflower *BoP2* locus seems to encompass both conditions; while retrotransposon insertion at the promoter -378 position of the wild type allele resulted in a low level of gene expression, transposon insertion at the promoter -373 position of the mutant allele resulted in a high level of gene expression. It still remains unclear how the promoter differences resulted in such a distinct phenotype, and further promoter analysis of the two alleles will be carried out to obtain more information.

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## CHAPTER 4

### ROLES OF BOP2 IN CONTROLLING ANTHOCYANIN BIOSYNTHESIS IN THE PURPLE CAULIFLOWER MUTANT

#### 4.1. ABSTRACT

A *Purple* gene mutation in cauliflower gave rise to an abnormal pattern of anthocyanin accumulation, resulting in an intense purple color in specific tissues. Through high-resolution genetic mapping, we have identified the MYB transcription factor, *BoP2*, which co-segregated with the *Purple* locus. *BoP2* exhibited tissue-specific expression consistent with the anthocyanin accumulation pattern in the mutant. In this chapter, we have confirmed that the *Purple* locus encoded *BoP2* through phenotypic complementation in *Arabidopsis* and cauliflower. Expression of the *BoP2<sub>pr</sub>* in transgenic *Arabidopsis* and cauliflower resulted in tissue-specific accumulation of anthocyanins. Comparison of the DNA sequences between the WT and mutant alleles suggested that the mutation might be caused by an abundance of R Response Elements (RRE) located in the promoter region of the *Purple* allele. Analysis of *Arabidopsis* transformants with different fragments of cauliflower *BoP2* promoter-GUS constructs revealed that the expression level of the *GUS* gene was correlated with the number of RREs. The *Purple* gene together with a bHLH transcription factor activated a subset of anthocyanin structural genes to produce the striking mutant phenotype. Transformation of the *BoP2* into *Arabidopsis* mutant lines for different bHLH transcription factors demonstrated that TT8, EGL3, and GL3 were all involved in the *BoP2* induced anthocyanin accumulation. Our results strongly suggest that the change of genomic context in the promoter of the *BoP2<sub>pr</sub>* is the genetic basis for the purple cauliflower mutant and that the increased expression in *BoP2* transcript levels accounts for the ectopic anthocyanin accumulation in the mutant plant.

## 4.2. INTRODUCTION

The activation of anthocyanin biosynthetic structural genes is regulated by the transcription factor complex WBM, which consists of a WD40 protein, basic helix-loop-helix proteins and MYB proteins (Koes et al. 2005, Vom Endt et al. 2002). In some cases, a single transcription factor has also been shown to activate the biosynthetic pathway (Grotewold et al. 1994). The detailed mechanism by which the transcription factors regulate anthocyanin biosynthesis has been studied in *Arabidopsis* (Gonzalez et al. 2008), a closely related species of cauliflower. However the combination of transcription factors and the transcriptional regulation of these transcription factors appear to be very complex. In *Arabidopsis*, three subgroup IIIb bHLH proteins, TT8, EGL3 and GL3, and several MYB proteins, such as PAP1, PAP2 and AtMYBL2 are involved in the WBM complex (Dubos et al. 2008, Gonzalez et al. 2008, Heim et al. 2003, Matsui et al. 2008, Ramsay and Glover 2005, Winkel-Shirley 2001). TT8, EGL3 and GL3 are responsible for anthocyanin and proanthocyanidin accumulation in different tissues (Baudry et al. 2006). The expression of bHLH and MYB proteins can be regulated by the WBM complex or by the transcription factor itself (Baudry et al. 2006, Espley et al. 2009), and the MYB proteins play different roles in the regulation of anthocyanin structural genes. The subgroup 6 R2R3-MYB proteins result tissue specific anthocyanin accumulation (Gonzalez et al. 2008) while the subgroup 4 R2R3-MYB proteins and R3-MYB proteins act as inhibitors of expression (Dubos et al. 2008, Hemm et al. 2001, Matsui et al. 2008). Moreover, even without the formation of the WBM complex, MYB proteins can work alone to activate anthocyanin biosynthesis in maize (Grotewold et al. 1994). It still remains unclear how exactly the transcription factor network acts in regulating anthocyanin biosynthetic structural genes and transcription factor expression.

The subgroup 6 R2R2-MYB in *Arabidopsis* and the orthologous genes are the key regulators of anthocyanin accumulation in many species. Over-expression of *PAP1* and *PAP2* in *Arabidopsis* induces anthocyanin accumulation in the entire plant tissues (Borevitz et al. 2000), while over-expression of *AtMYB113* and *AtMYB114* results in anthocyanin accumulation in the embryos and young seedlings (Gonzalez et al. 2008), suggesting functional differences between the different R2R3-MYB proteins within this subgroup. *PAP2*, *MYB113* and *MYB114* in *Arabidopsis* appear in tandem on chromosome one (TAIR, [www.arabidopsis.org](http://www.arabidopsis.org)), and despite the wealth of knowledge concerning *PAP1* in regulating the anthocyanin biosynthesis, few studies have focused on *PAP2*, *MYB113* and *MYB114* (Gonzalez et al. 2008). Previously, we identified four *PAP* orthologs in cauliflower, three of which are located in tandem. *BoP2* represents the gene conferring high levels of anthocyanin accumulation in the purple cauliflower and is one of the three *MYB* genes in tandem.

Although significant progress has been made in our understanding of anthocyanin biosynthesis and regulation, only limited information is available on the transcriptional regulation of the transcription factors involved. The expression of *TT8* can be regulated by itself, as well as by *EGL3* and *GL3* in different tissues via WMB complex regulation (Baudry et al. 2006). A retrotransposon insertion in the grape *MYBA1* promoter region results in white berry skin (Kobayashi et al. 2004), whereas in apple, the mini satellite-like structure in the promoter of *MYB10* leads to autoregulation of the gene (Espley et al. 2009).

We have identified the R2R3-MYB transcription factor *BoP2* with an autonomous DNA transposon insertion at the -373 position, which generates several bHLH protein recognition sites named RRE (R response elements) in its promoter. The RREs has been demonstrated to be recognized by a bHLH in activating the expression of the structural gene, *CHS* (Hartmann et al. 2005). In the previous chapter,

we showed that the number of RRE elements is correlated with the level of expression of *BoP2*. The promoter structure of *BoP2* suggests that the expression of *BoP2* is regulated by a bHLH protein. Our previous *Arabidopsis* transformation results demonstrated that TT8, EGL3, and GL3 were all involved in *BoP2* induced anthocyanins accumulation.

### **4.3. MATERIALS AND METHODS**

#### ***4.3.1. Plasmid construction and plant transformation***

To generate the constructs for phenotypic complementation, genomic DNA of both *BoP2* alleles, including 1755 or 1061 bp of promoter sequence for the mutant and wild type allele, respectively, were amplified using primers (65, 67, and 66, 67 in Table A1.1). The amplified products were cloned into the pCR2.1 vector (Invitrogen, Carlsbad, CA) and then into the pCAMBIA 1300 vector (CAMBIA, Australia) with an additional NOS terminator from the pBI121 vector attached at the end of *BoP2* to produce *P2<sub>Pr-1755</sub>:BoP2<sub>Pr</sub>* and *P2<sub>WT-1061</sub>:BoP2<sub>WT</sub>*. To generate the over-expression constructs, *BoP2* genomic DNA for the coding region of both alleles was amplified (67 and 68), cloned into the pCR2.1 vector, and inserted into the pCAMBIA 1300S to produce *35S:BoP2<sub>Pr</sub>* and *35S:BoP2<sub>WT</sub>*. To create the GUS constructs, the promoters of the *BoP2* wild type allele (-378 and -1061 bp), and mutant allele (-373, -1079, and -1755) were amplified using primers 69 and 70, 69 and 73, 69 and 70, 69 and 71 and 69 and 72, respectively (Table A1.1), and cloned into pCR2.1. Promoter regions were then inserted into the pSG506 vector (Lu et al. 2006) between the *NotI* and *NcoI* sites to fuse the promoter regions to GUS. The fragments were then subcloned into the pCAMBIA 1300 vector to produce various promoter-GUS constructs. All constructs were sequenced to confirm the cloning sites.

The constructs and vector-only controls were electroporated into the *Agrobacterium tumefaciens* strain, GV3101. Cauliflower cultivar Fremont (Harris Seeds, Rochester, NY) and *Arabidopsis thaliana* Ecotype Columbia wild type, as well as *tt8* (CS111), *ttg1* (CS89), *gl3* (CS66), *egl3* (SALK 077439), and *gl3egl3* (CS6516) mutant lines (TAIR, [www.arabidopsis.org](http://www.arabidopsis.org)) were used for genetic transformation. The plasmids constructs were transformed into *Arabidopsis* using the floral dip method (Clough and Bent 1998). The T1 seeds were screened on a medium containing 50 mg hygromycin, 100 mg Timentin, 2.3g MS salt and 1% sucrose. Seeds on the medium were placed at 4°C for 2 days in the dark for vernalization, and then moved to room temperature for five days in the dark. Positive transformants with long hypocotyls were selected and transferred to soil. Pictures of the *Arabidopsis* T2 young seedlings, seeds and GUS histochemical stained tissues were taken directly under an Olympus SZX12 stereo microscope.

Constructs of *35S:BoP2<sub>Pr</sub>* and *35S:BoP2<sub>WT</sub>* as well as *P2<sub>Pr-1755</sub>:BoP2<sub>Pr</sub>* and *P2<sub>WT-1061</sub>:BoP2<sub>WT</sub>* were transformed into the cauliflower cultivar Fremont, using the *Agrobacterium tumefaciens*-mediated tissue culture method (Cao et al. 2003, Lu et al. 2006). Positive transformants were confirmed by PCR amplification of the selective markers. The *Arabidopsis* and cauliflower T2/T1 lines were grown in a greenhouse under 14-h-light/10-h-dark cycle at 23°C for further characterization.

#### **4.3.2. RNA analysis**

Total RNA samples from the transformed cauliflower and *Arabidopsis* young leaves tissues were extracted using Trizol reagent. cDNA was then synthesized from the total RNA (5µg) using the Superscript III reverse transcriptase (Invitrogen, Carlsbad, CA), with the concentrations in different samples normalized as described in Chapter 2. Gene specific primers were designed based on the sequences in both *Arabidopsis*

(TAIR, [www.arabidopsis.org](http://www.arabidopsis.org)) and *Brassica Oleracea* (TIGR, [www.tigr.org/tdb/e2k1/bog1](http://www.tigr.org/tdb/e2k1/bog1)) databases (Yuan et al. 2009). Gene specific primers of *CHS* (1 and 2), *CHI* (3 and 4), *F3H* (5 and 6), *F3'H* (7 and 8), *DFR* (9 and 10), *LDOX* (11 and 12), *UGT* (15 and 16), *BoEGL3* (33 and 34), *BoTTG1* (19 and 20), *BoTT8* (23 and 24), *BoP2* (37 and 38), *AtGL3* (47 and 48), *AtEGL3* (45 and 46), *AtTT8* (43 and 44), *PAP1* (49 and 50), *PAP2* (51 and 52), *AtMYB113* (53 and 54), and *AtMYB114* (55 and 56) (Table A1.1) were used to amplify the target genes with optimized cycles of amplification.

#### **4.3.3. *GUS* analysis**

Histochemical analysis of the *GUS* expression in transgenic plants was carried out using 5-bromo-4-chloro-3-indolyl  $\beta$ -D-glucuronide (X-gluc) as the substrate following a previously described procedure (Jefferson et al. 1987). Quantitative analysis of the *GUS* activity in transformants expressing the *GUS* gene under different *BoP2* endogenous promoter controls were carried out using a fluorometric assay (Blazquez 2007) with slight modifications. Entire plants of four-week old *Arabidopsis* were used for the assay. Plant tissues (25 mg) were extracted in a 100  $\mu$ l *GUS* extraction buffer containing 10 mM EDTA pH8.0, 0.1% SDS, 50 mM sodium phosphate pH7.0, 0.1% Triton x-100, 10 mM  $\beta$ -mercaptoethanol (freshly added), and 25  $\mu$ g/ml PMSF. The crude extract (5  $\mu$ l) was added to a 450 $\mu$ l *GUS* extraction buffer containing 1mM 4-MUG, and incubated at 37°C. Aliquot of the reaction mixture (20 $\mu$ l) was added to a 180  $\mu$ l stop solution (1M sodium carbonate) every 10 min. The fluorescence was then measured by the Fluorolite 1000 fluorometer (DYNEX technologies, Chantilly, VA) at the excitation wavelength of 365 nm and emission wavelength 450 nm.



#### **4.3.4. Yeast one and yeast two hybrid analysis**

To construct the plasmids for yeast two-hybrid assays, the full length cDNA of *BoTTG1* (74 and 75), *BoTT8* (76 and 77), *BoP1* (78 and 79), *BoP2<sub>Pr</sub>* (80 and 81), *BoP2<sub>WT</sub>* (80 and 81), *BoP3* (82 and 83), and *BoP4* (83 and 84) (Table A1.1) were amplified and cloned downstream of GAL4<sup>AD</sup> and GAL4<sup>BD</sup>, respectively, in the pAD-GAL4-2.1 and pBD-GAL4 Cam vectors (Stratagene, La Jolla, CA). All plasmid constructs were verified by sequencing.

The two-hybrid assay was essentially performed following the procedure as described in the Stratagene instruction manual for the GAL4 Two-Hybrid Phagemid Vector Kits. The AD constructs were then transformed into the yeast strain PJ69-4 $\alpha$  and the BD constructs into strain PJ69-4a. They were selected on synthetic dextrose plates lacking Leu or Trp, respectively, for positive colonies. Cultures from single colonies of each transformant were systematically mated with one another by adding 100  $\mu$ l of each cell culture to a 2 ml YPD medium, incubating overnight at 30°C, and plating on a -Leu/-Trp medium. They were then sub-cultured, spotted on a -Leu/-Trp/-Ade medium with the same concentration of cells, and incubated at 30°C for up to seven days to examine the protein-protein interactions. To obtain the same cell concentrations, the cell density was measured at 600 nm. Cell cultures with different times of dilution were also spotted on a -Leu/-Trp/-Ade medium to examine the protein-protein binding capacity.

To construct the plasmids for yeast one-hybrid assays, the -838 *BoTT8* (93 and 94), -1755 *BoP2<sub>Pr</sub>* (95 and 96) and -1061 *BoP2<sub>WT</sub>* (95 and 97) promoter regions were amplified using specific primers and cloned into the pLacZi2u one hybrid vector (Wang Lab) with the *SacI* and *SpeI* modified at the 5' and 3' ends, respectively. cDNA of *BoTT8* (98 and 99), *BoP2<sub>Pr</sub>* (100 and 101), *BoP2<sub>WT</sub>* (100 and 101) were amplified using gene specific primers with *XhoI* and *EcoRI* modified to clone into the GAL1

downstream site in the pJG4-5 vector. Vectors with different combinations were transferred together into the EG486 yeast strain for detection of possible *LacZ* expression on a SD media with -Trp/-Ura dropout plate with 20 mg X-Glu, as outlined in the MATCHMAKER One-Hybrid System user manual (Clontech, Mountain View, CA).

## 4.4. RESULTS

### 4.4.1. Functional complementation of the purple mutant phenotype by *BoP2* in *Arabidopsis* and cauliflower

Previously, we showed that *BoP2* co-segregates with the *Purple* locus controlling anthocyanin synthesis in purple cauliflower (Figure 3.9). To confirm that *BoP2* represented the *pr* gene, we conducted a functional complementation of the *pr* mutant phenotype in wild type *Arabidopsis* and cauliflower. Genomic DNA for the *BoP2* alleles from wild type and mutant plants as well as coding region DNA for both alleles under the control of CaMV35S were introduced into wild type *Arabidopsis* and white cauliflower. Over 50 independent transgenic *Arabidopsis* lines were generated for each construct. Over-expression of either the wild type or mutant gene under the control of CaMV35S promoter (*35S:BoP2<sub>pr</sub>* and *35S:BoP2<sub>WT</sub>*) resulted in the production of dark purple transgenic plant with high levels of anthocyanin accumulation in *Arabidopsis* (Figure 4.1.D). The pigment was accumulated throughout the entire transgenic plants, including the leaves, roots, seeds, flower buds, and flower petals (Figure 4.1). Similarly, when the *35S:BoP2* gene constructs were introduced into wild type cauliflower plants, pigment appeared throughout the entire transgenic plant (Figure 4.2 and Figure 4.3). However, cauliflower transformation appeared to result in less anthocyanin accumulation from the wild type construct compared to the mutant construct (Figure 4.3.C). These results suggested that both



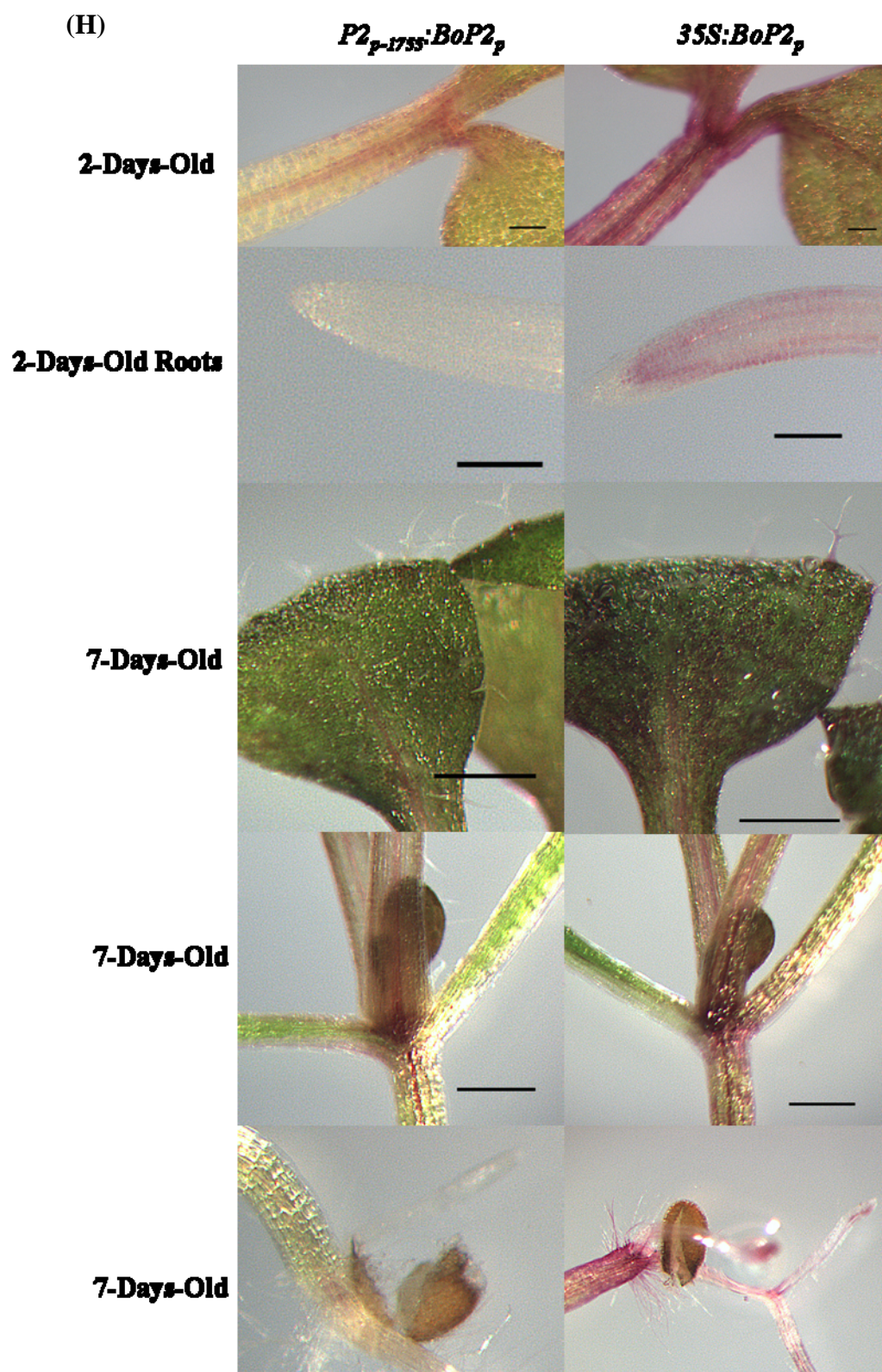
**Figure 4.1.** *Arabidopsis* transgenic lines with different *BoP2* constructs in Col WT seeds (A), 2-day old seedlings (B), 7-day old seedlings (C), 3-week old plants (D), young flower buds (E), flowers (F), and old siliques (G) Detailed view of *P2<sub>Pr</sub>-175S:BoP2<sub>Pr</sub>* (left) and *35S:BoP2<sub>Pr</sub>* (right) transformed plants (H). Scale bars: A and H 2-Days-Old, 0.1 mm, B and H 7-Days-Old, 0.5 mm. 1: Vector only control, 2: *P2<sub>Pr</sub>-175S:BoP2<sub>Pr</sub>*, 3: *P2<sub>WT</sub>-106I:P2<sub>WT</sub>*, 4: *35S:BoP2<sub>Pr</sub>*, 5: *35S:BoP2<sub>WT</sub>*, 6: *PAPID*

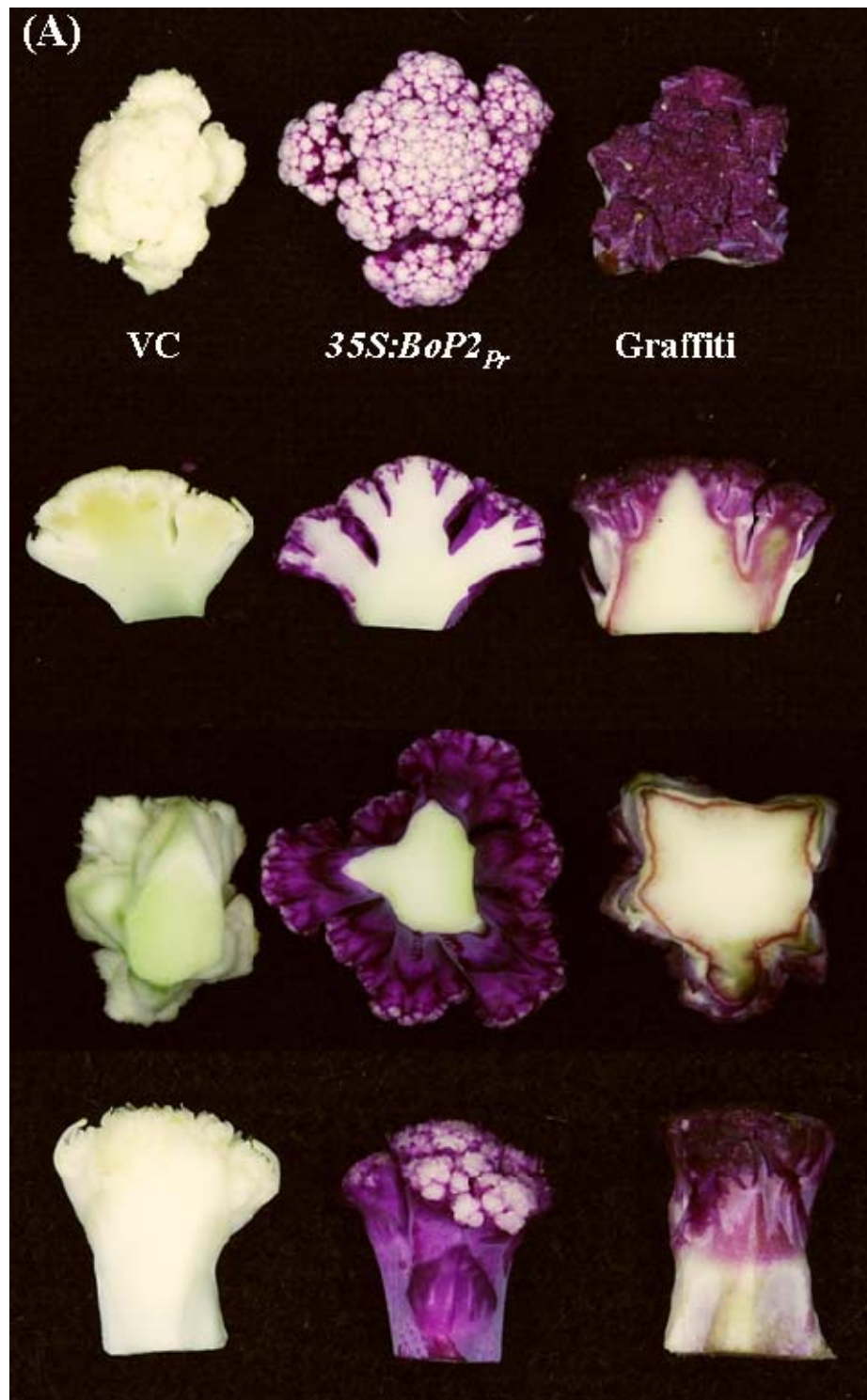
Figure 4.1. continued





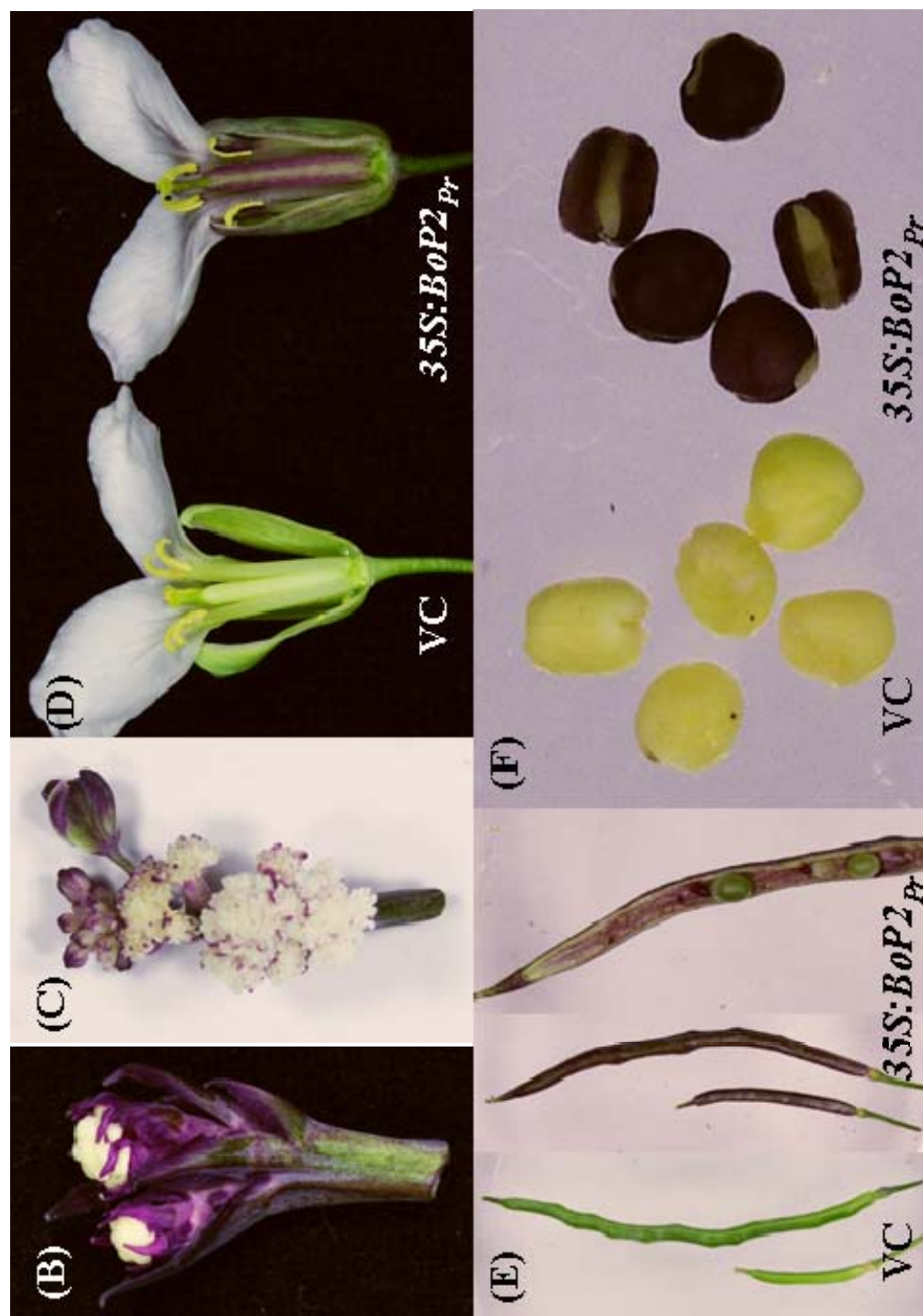
Figure 4.1. continued





**Figure 4.2.** Cauliflower transformants expressing with *35:BoP2<sub>Pr</sub>* construct. Curds (**A, B**), young to old flower buds (**C**), flowers (**D**), young and old siliques (**E**) and seeds (**F**). VC: Vector only control

Figure 4.2. continued



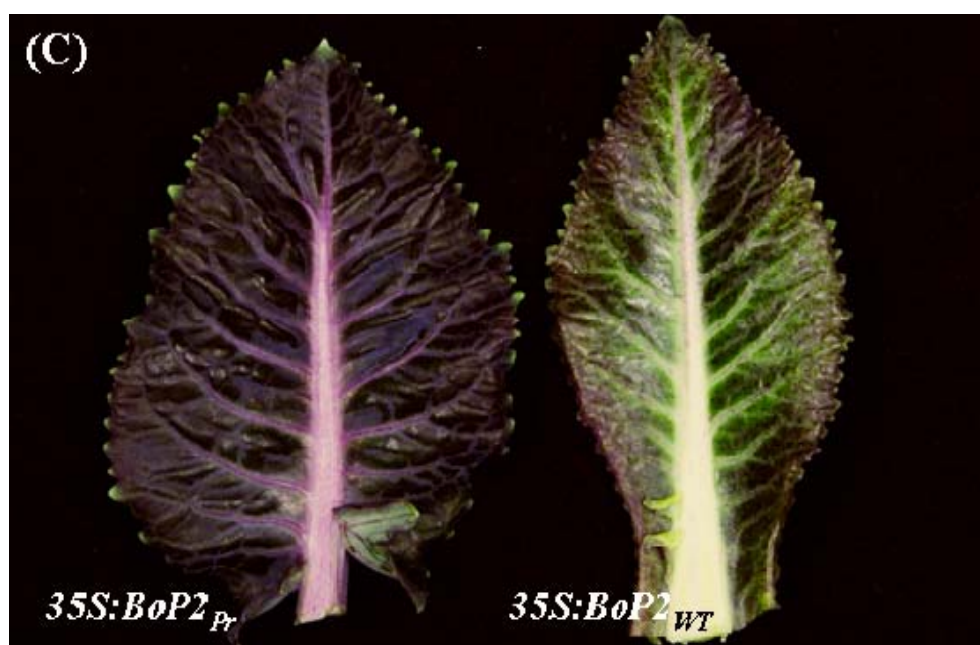




**Figure 4.3.** Leaves (A) and curds (B) of different *BoP2* transgenic lines. The  $35S:BoP2_{WT}$  transformants accumulated less anthocyanins compared to the  $35S:BoP2_{Pr}$  transgenic lines (C).



Figure 4.3. continued



*BoP2<sub>pr</sub>* and *BoP2<sub>WT</sub>* were functional alleles. Interestingly, in comparison with the purple mutant, the top curd tissue of these over-expression lines appeared to be unaffected and remained white (Figure 4.2), suggesting that the promoter region of *pr* controlled its tissue specific accumulation of anthocyanins in the purple cauliflower mutant.

In contrast, transgenic *Arabidopsis* expressing *BoP2* alleles under the control of their endogenous promoter exhibited different phenotypes. As with the vector only control, the *BoP2<sub>WT</sub>* transgenic plants exhibited no or very low levels of pigmentation (Figure 4.1, #3). However, the *BoP2<sub>pr</sub>* transformants exhibited tissue-specific anthocyanin accumulation (Figure 4.1, #2). The purple anthocyanin pigments were mainly accumulated in the young tissues of *Arabidopsis* including young leaves, young flower buds, as well as in the seeds, but not in mature leaves, flower petals, and mature siliques (Figure 4.1, #2). Likewise, a similar pattern of anthocyanin accumulation was also observed in transgenic cauliflower plants expressing the *BoP2<sub>pr</sub>* allele (Figure 4.2 and Figure 4.3). Ectopic expression of the *BoP2<sub>pr</sub>* in both transgenic *Arabidopsis* and cauliflower induced tissue-specific anthocyanin accumulation, which recapitulated the tissue-specific anthocyanin accumulation pattern in the *pr* mutant plants. Thus, these results further confirmed the successful cloning of the *pr* gene.

#### ***4.4.2. pr specifically regulates a variety of genes involved in anthocyanin accumulation***

A number of late anthocyanins biosynthetic pathway structural genes and the transcription factor *BoTT8*, along with *BoP2*, were expressed highly in the purple mutant (Figure 2.7 and Figure 2.8). To investigate whether expression of the *BoP2* in transgenic *Arabidopsis* and cauliflower also caused the specific activation of *TT8* and

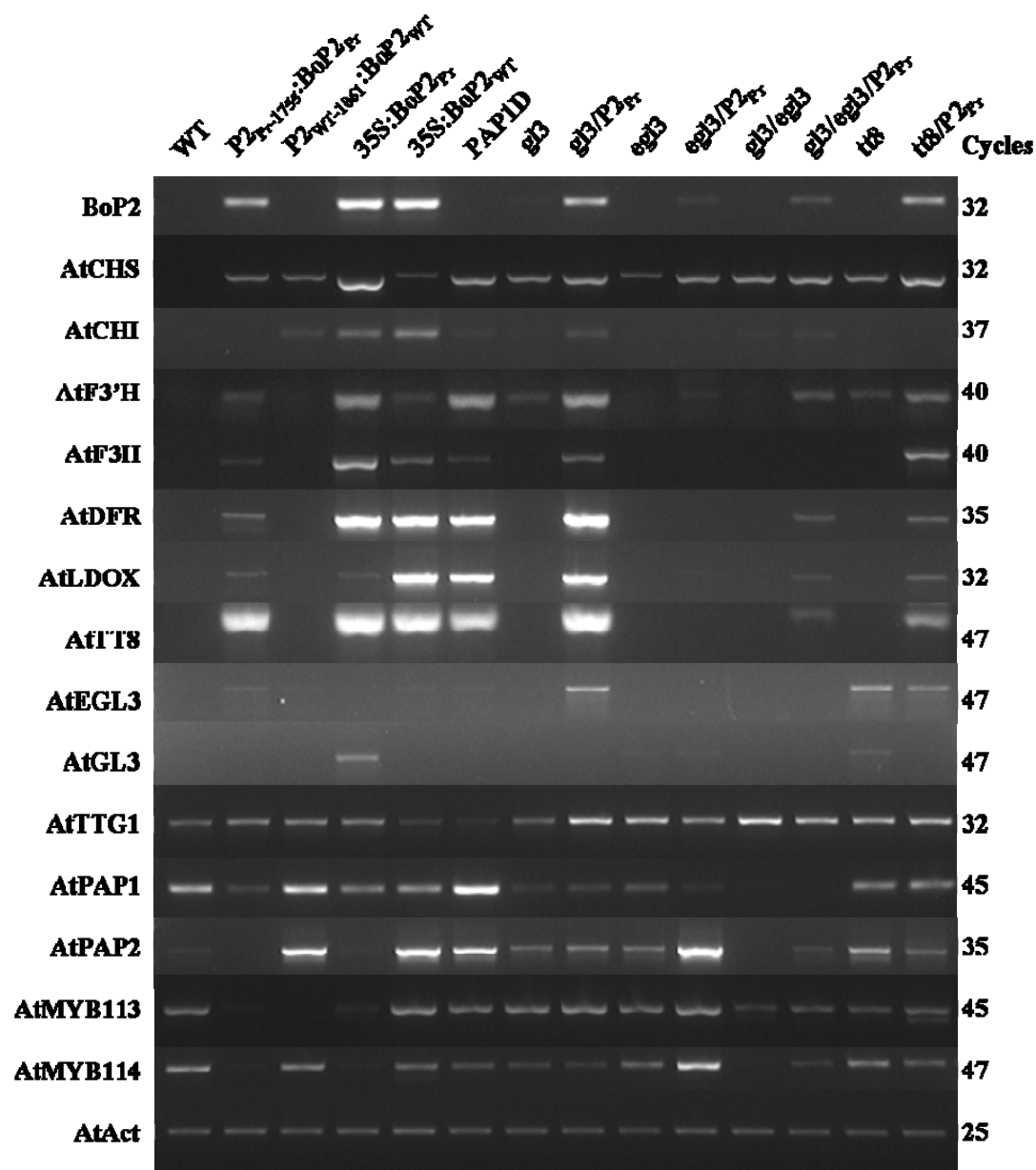
the anthocyanin late structural genes, the transcript levels of those anthocyanin structural and regulatory genes from young leaves of the *BoP2* transgenic lines were analyzed. As shown in Figure 4.4 and Figure 4.5, the late structural genes *F3'H*, *DFR*, *LDOX*, and the transcription factor *TT8*, were expressed highly in all *35S:P2* as well as *P2<sub>Pr-1755</sub>:BoP2<sub>Pr</sub>* lines in comparison with the vector only control and *P2<sub>WT-1061</sub>:BoP2<sub>WT</sub>* lines for both transgenic *Arabidopsis* and cauliflower. The early structural genes, however, exhibited no specific up-regulation by *BoP2* over-expression. These results clearly demonstrated that *pr* specifically activated *BoTT8* and the late structural genes in controlling anthocyanin biosynthesis in the purple cauliflower mutant.

#### **4.4.3. *BoP2* is expressed highly in young tissues**

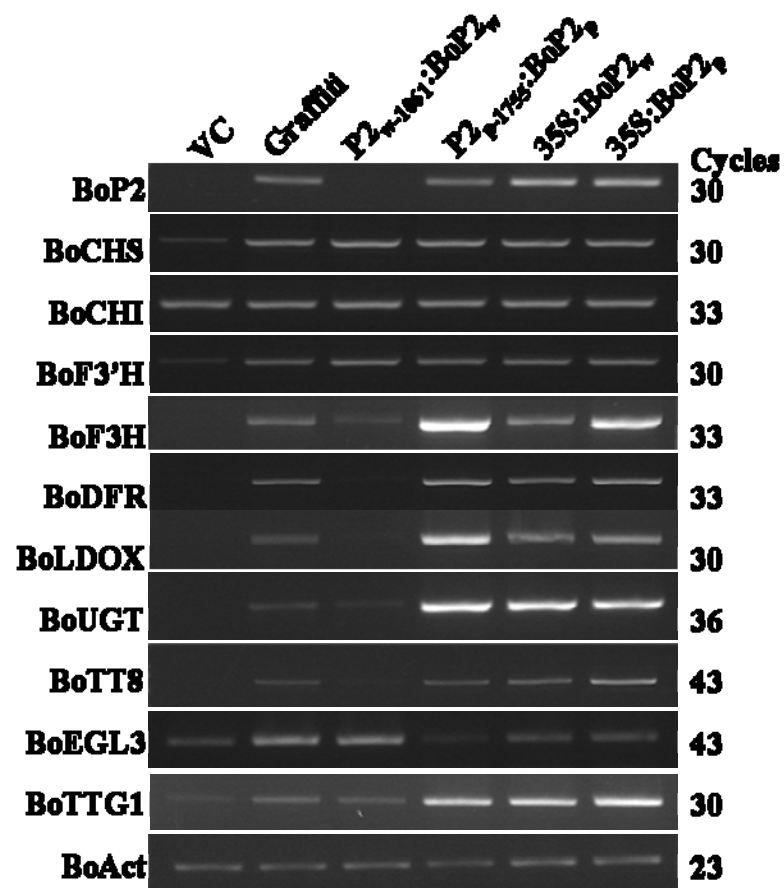
In order to gain further insight into the expression pattern of *BoP2*, the promoter of *BoP2<sub>Pr</sub>* (-1755 bp) was fused to the  $\beta$ -D-glucuronidase (*GUS*) gene and introduced into *Arabidopsis* plants. Histochemical localization of GUS activity throughout plant development for five independent transgenic lines was analyzed and the representative images are shown in Figure 4.6. Although the GUS activity was observed in all tissues, it was present at high levels in young tissues such as the young leaves and flower buds, rather than in the flower petals. Moreover, the GUS activity was expressed highly in vascular bundles of hypocotyles. The overall *GUS* expression pattern in the *Arabidopsis* transformants was the same as the *BoP2* transcript pattern in the cauliflower mutant.

#### **4.4.4. *Cis-elements* in the *BoP2<sub>Pr</sub>* promoter affect high levels of anthocyanin accumulation in the *pr* mutant**

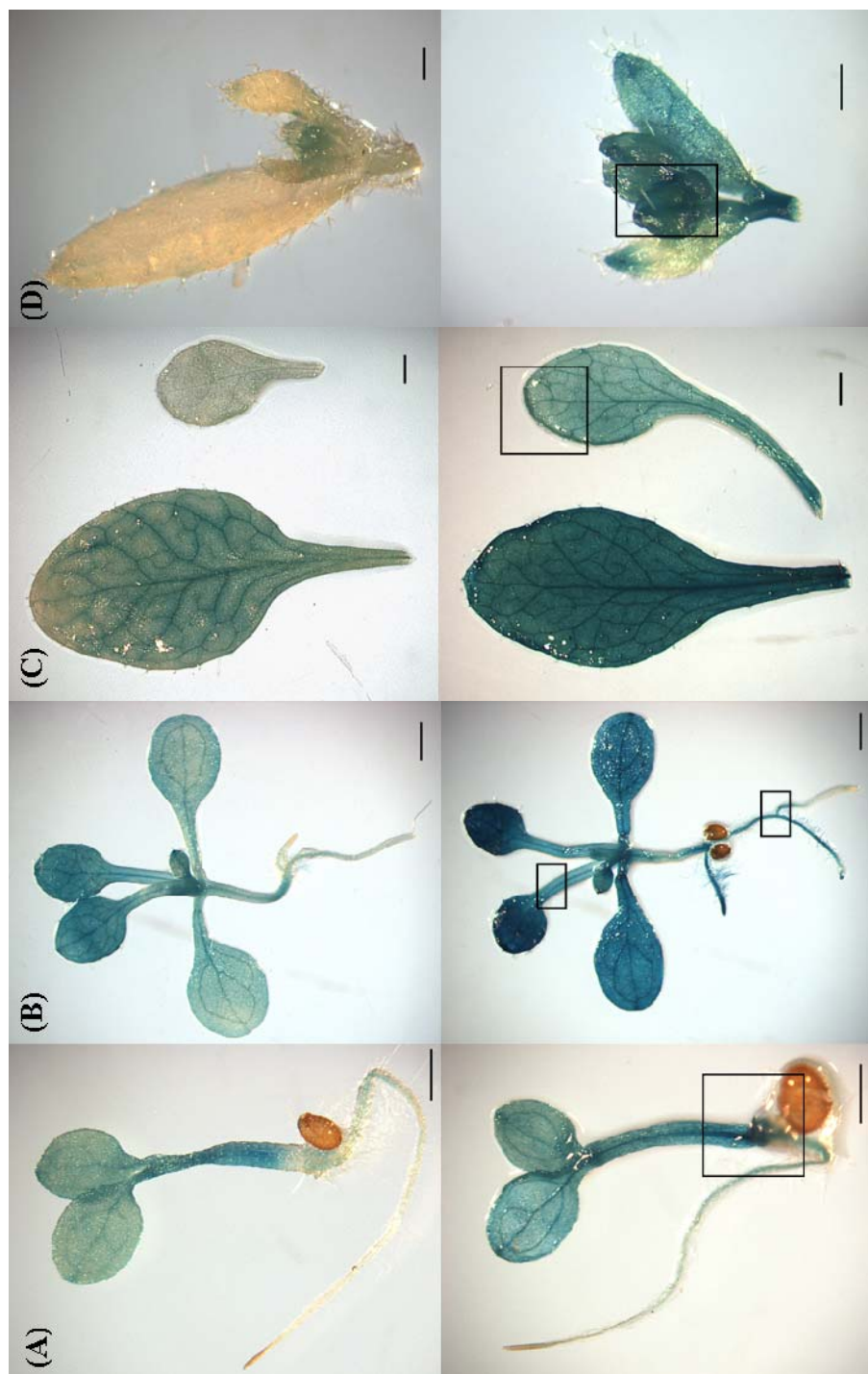
*BoP2<sub>Pr</sub>* was expressed highly in the *pr* mutant and shared high sequence homology in the coding region with *BoP2<sub>WT</sub>*. Thus, it has been hypothesized that the mutation in the



**Figure 4.4.** Gene expression pattern of different *BoP2* *Arabidopsis* transgenic lines in young leaves.

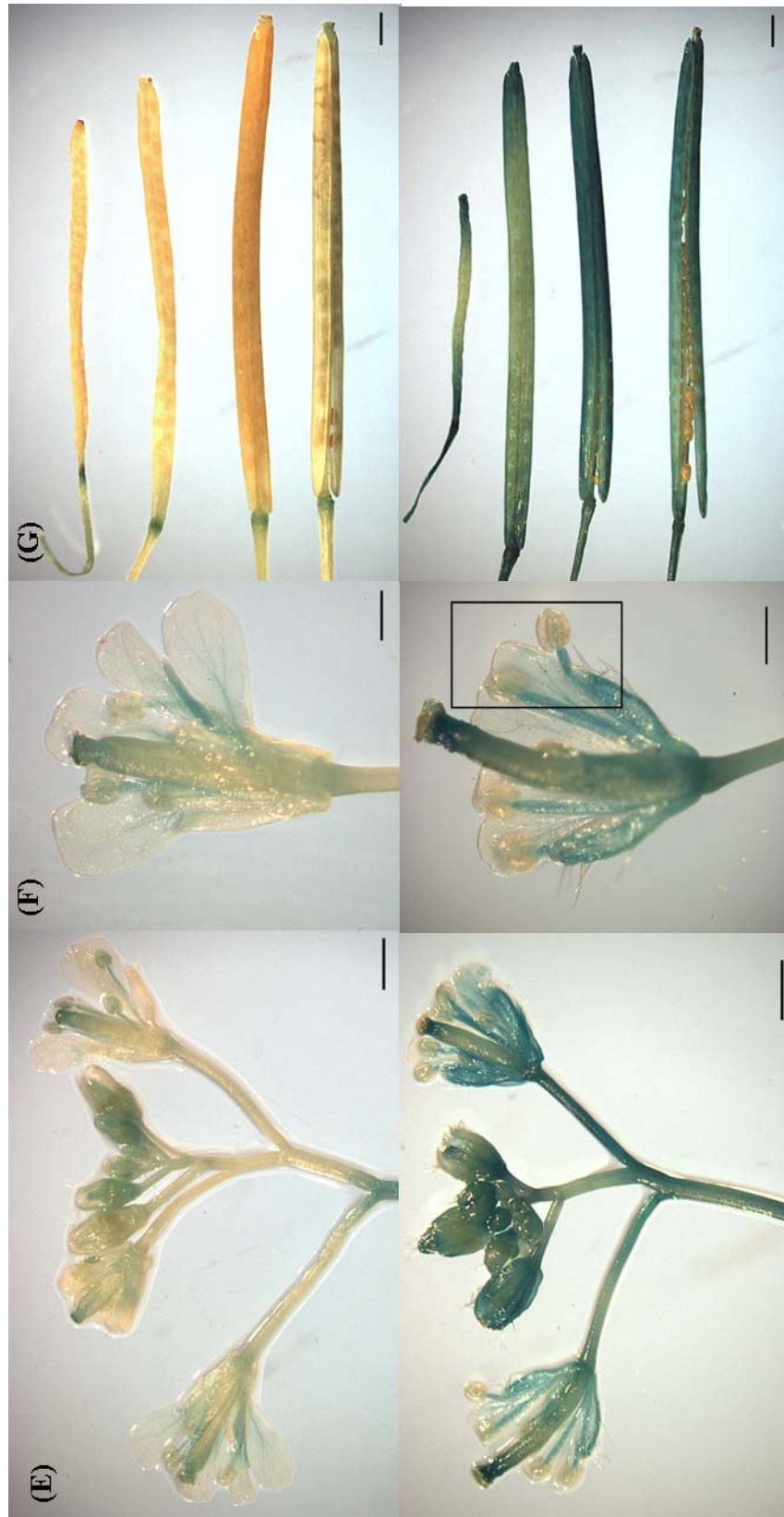


**Figure 4.5.** Gene expression pattern of different *BoP2* cauliflower transgenic lines in young leaves.



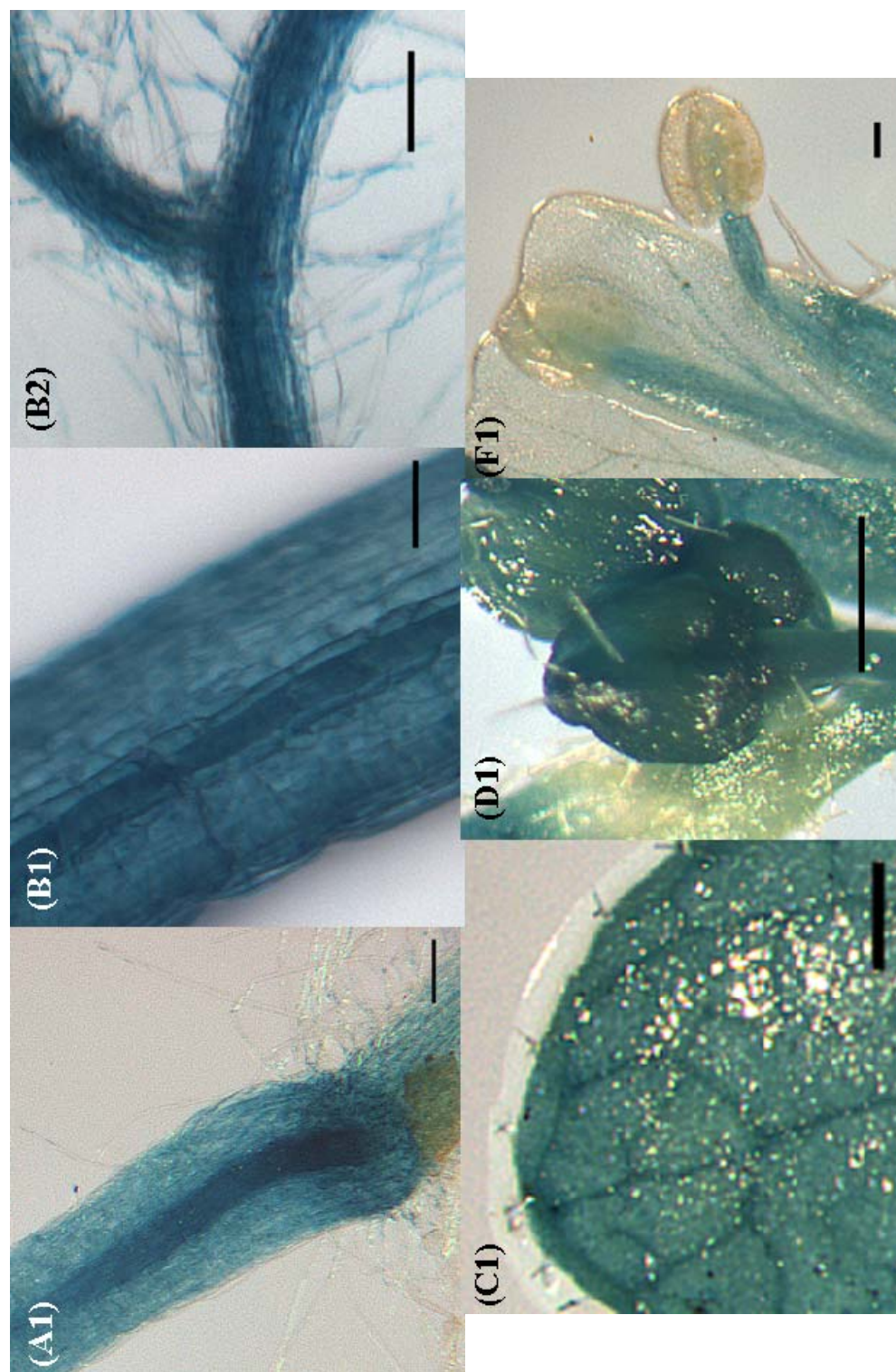
**Figure 4.6.** GUS expression pattern under *BoP2* wild type (upper panel) and mutant (lower panel) promoter control in 2-day old seedling (A), 7-day old seedling (B), young to old leaves (C), young flower buds (D), flowers and flower buds (E), flower (F), and young to old siliques. Scale bars: A, D, F, C1 and D1, 500  $\mu$ m; B, C, E and G 1 mm; A1, B1, B2 and F1 100  $\mu$ m.

**Figure 4.6. continued**





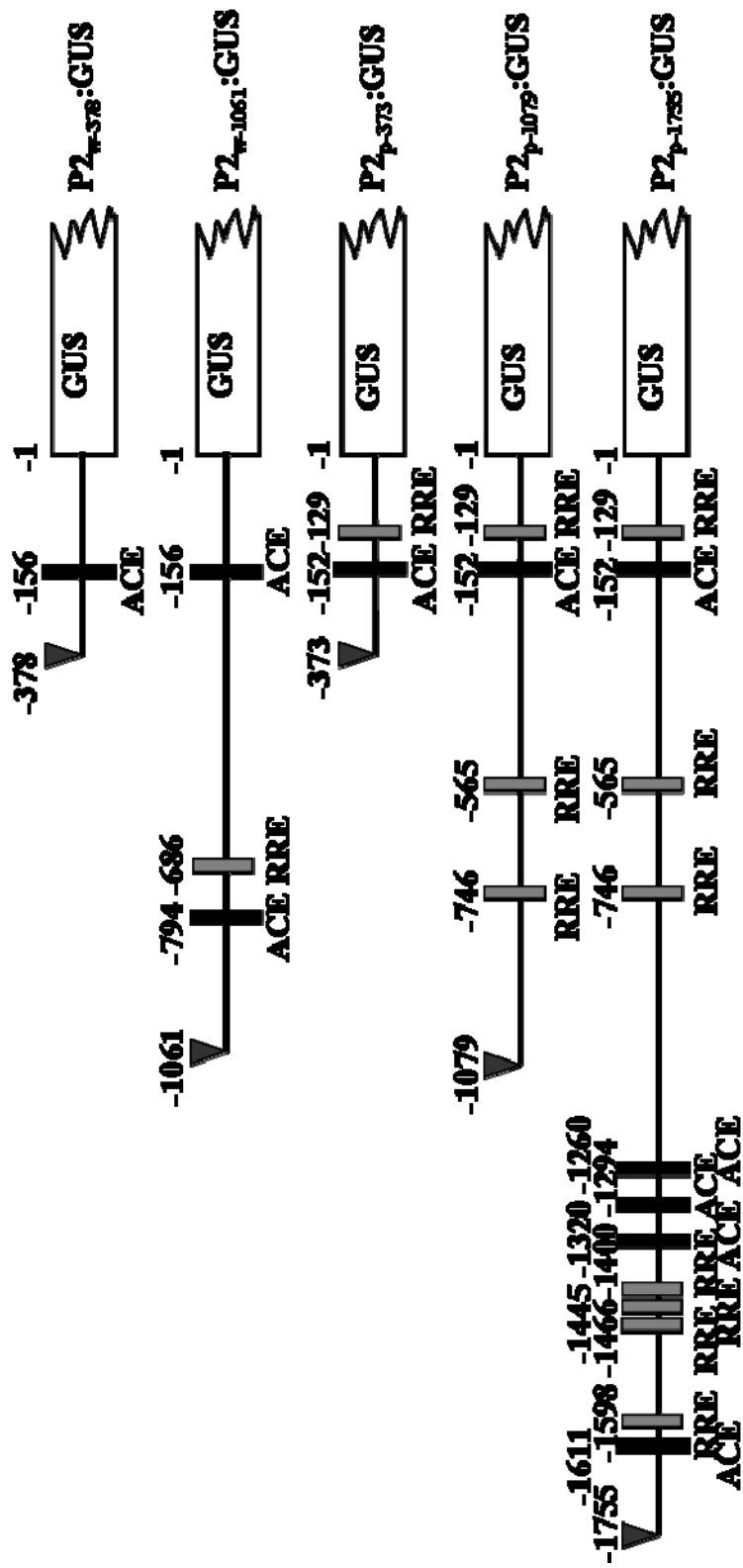
**Figure 4.6. continued**



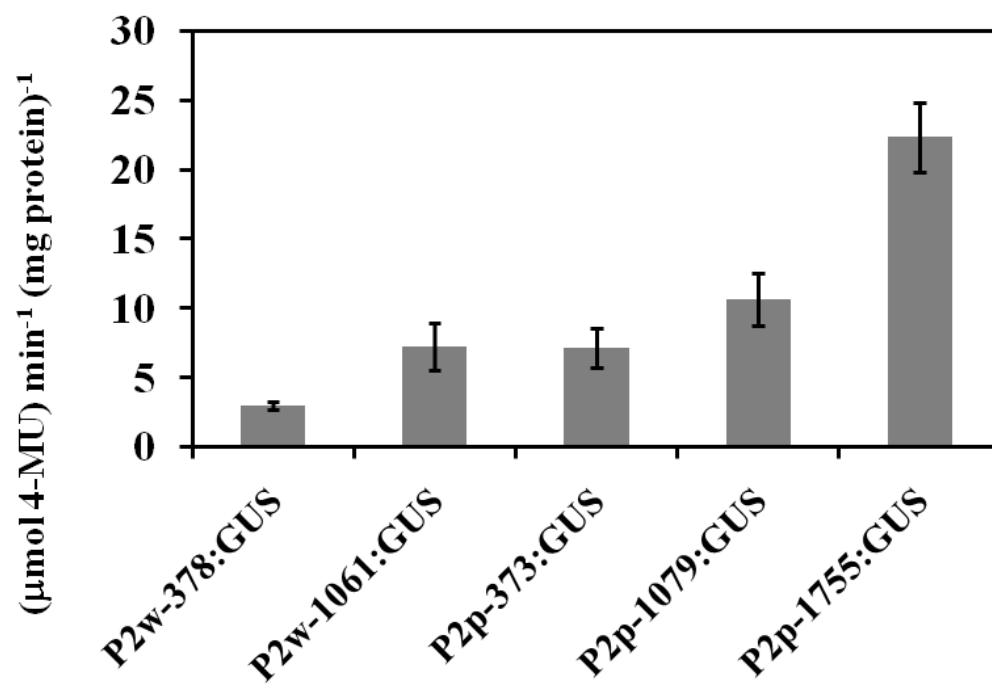


promoter sequence controls the expression of *BoP2* in regulating anthocyanin accumulation in the *pr* mutant. To test this hypothesis, GUS constructs under the control of different promoter regions of *BoP2* were transformed into *Arabidopsis*. The constructs included *P2<sub>W-378</sub>:GUS*, *P2<sub>W-1061</sub>:GUS*, *P2<sub>P-373</sub>:GUS*, *P2<sub>P-1079</sub>:GUS*, and *P2<sub>P-1755</sub>:GUS* (Figure 4.7). The promoter regions were chosen based on the retrotransposon or transposon insertion sites and the transposase site. GUS activity for transformants expressing these constructs was measured and is shown in Figure 4.8. The *P2<sub>P-1755</sub>:GUS* transformants exhibited the highest GUS activities (22.36  $\mu\text{mol 4-MU min}^{-1} \text{mg protein}^{-1}$ ). Both the *P2<sub>P-373</sub>:GUS* and *P2<sub>P-1079</sub>:GUS* transformants exhibited significant higher GUS activities than the comparable *P2<sub>W-378</sub>:GUS* and *P2<sub>W-1061</sub>:GUS* transgenic lines. These results indicated that the promoter of the mutant allele was responsible for the over-expression of *BoP2*.

The promoter sequences between the -378/-373 regions of *Pr* and *pr* share high sequence identities except for 7 single nucleotide differences between the two alleles and an extra TATA box in the wild type allele (Figure 4.9). Detailed analysis of these sequences revealed that changes of single nucleotides in the mutant sequence resulted in the introduction of a *cis*-acting element for the bHLH recognition site RRE (5'-CANNTG-3'), also known as the E-box, in the promoter of the mutant allele. The RRE was previously found in the promoter region of the anthocyanin biosynthetic structural gene, *CHS* (Hartmann et al. 1998, Hartmann et al. 2005), together with ACE, a bZIP protein recognition site, and MRE, a MYB protein recognition site, in close vicinity. Examination of the *BoP2* promoter sequences revealed the presence of different numbers of the RRE *cis*-elements in the GUS constructs. We found a number of ACEs near the RREs in the *BoP2* promoter, and although several MYB recognition sites were found in the vicinity of the RREs, no identical types of MREs for *CHS* were found in cauliflower. Figure 4.7 demonstrates schematically where the RREs and



**Figure 4.7.** Positions of RREs and ACEs in different GUS constructs. The bent arrow indicates the start side of each GUS construct.



**Figure 4.8.** The GUS activities under the controls of different *BoP2* promoters. The activities from left to right are 2.96, 7.22, 7.13, 10.65 and 22.36  $\mu\text{mol 4-MU} \text{ min}^{-1} \text{ mg protein}^{-1}$  respectively.

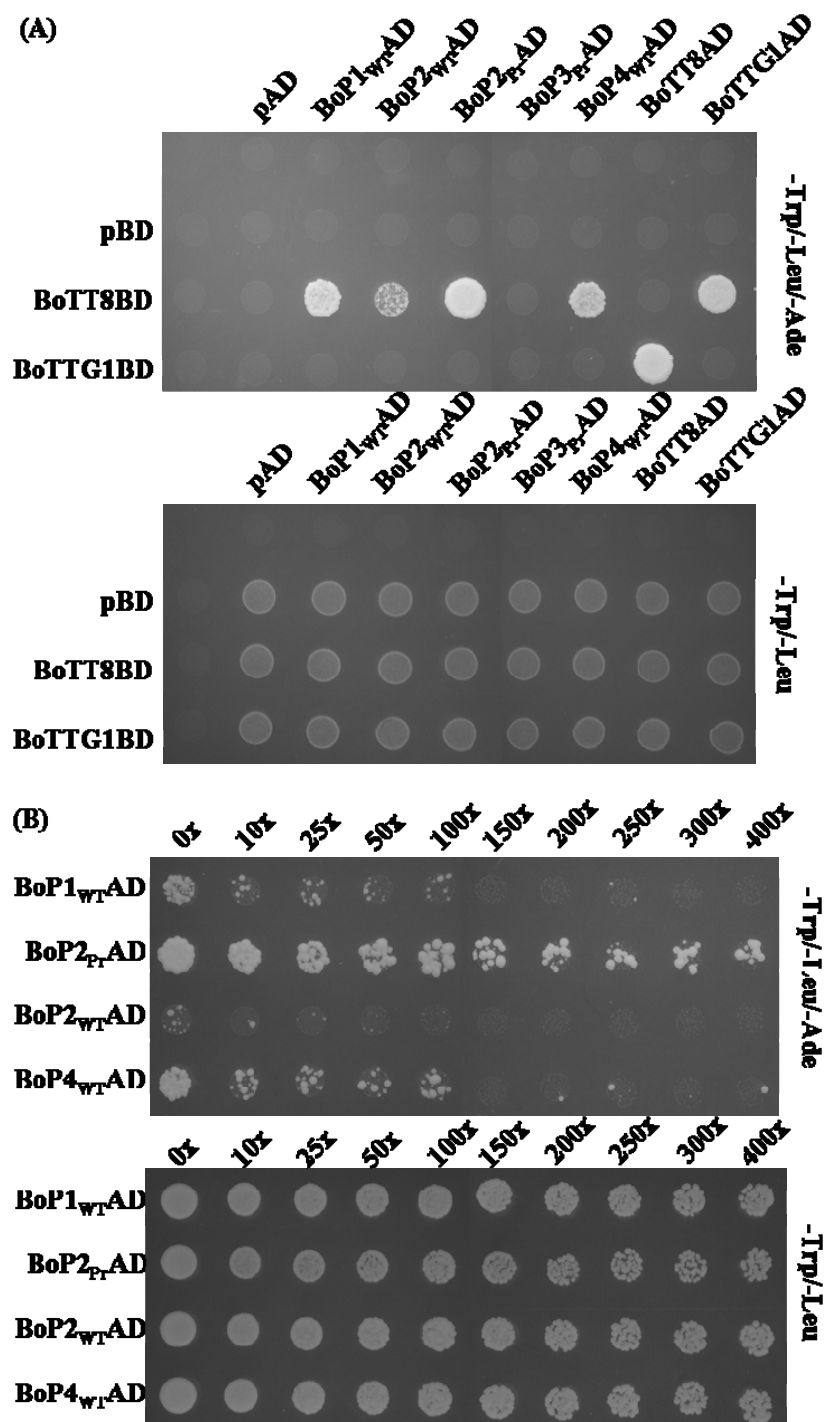


ACEs were located in the *BoP2* promoter. The promoter activity appears to be correlated with the numbers of RREs in the promoter region. Thus, our results demonstrated that the introduction of the RRE may play an important role in regulating the anthocyanin *MYB* gene expression.

#### ***4.4.5. Role of BoP2 in the WD40-MYB-bHLH transcriptional regulatory network***

PAP1, AtTT8 and AtTTG1 form a complex regulating the expression of anthocyanin biosynthetic genes (Broun 2005, Zimmermann et al. 2004). To better understand the role of BoP2 in interacting with other key transcriptional regulators to regulate anthocyanin biosynthesis in purple cauliflower, we performed a yeast two hybrid assay. Seven regulators were fused to the GAL4 activation domain (AD) and DNA binding domain (BD). The *BoP* genes were found to self-activate when they were fused to GAL4-BD. Thus, the *BoP* genes in the AD constructs were used to examine the protein-protein interactions. Different combinations of these fusion proteins were tested for the expression of the reporter, Ade2 (Figure 4.10.A). BoP proteins were found to bind only to BoTT8. BoP2<sub>Pr</sub> exhibited stronger binding activity to BoTT8 than other BoP WT proteins, and BoP3 exhibited no binding activity to BoTT8. Interestingly, BoP2<sub>WT</sub> exhibited much weaker binding activity to BoTT8 than BoP2<sub>Pr</sub> when similar numbers of cells were used (Figure 4.10.B), suggesting that the two amino acid changes (Figure 3.10) in the mutant protein might also have resulted in the activity differences between these two proteins. BoTTG1 only interacted with BoTT8, and not with BoP proteins. Unlike AtTT8 (Matsui et al. 2008), BoTT8 could not form a homodimer in this transcriptional complex (Figure 4.10.A). Therefore, BoTT8 may act differently from AtTT8 in regulating the anthocyanin biosynthesis.

Based on the results of the promoter analysis, we hypothesized that bHLH proteins may be involved in regulating *BoP2* expression. In apple, the expression of a

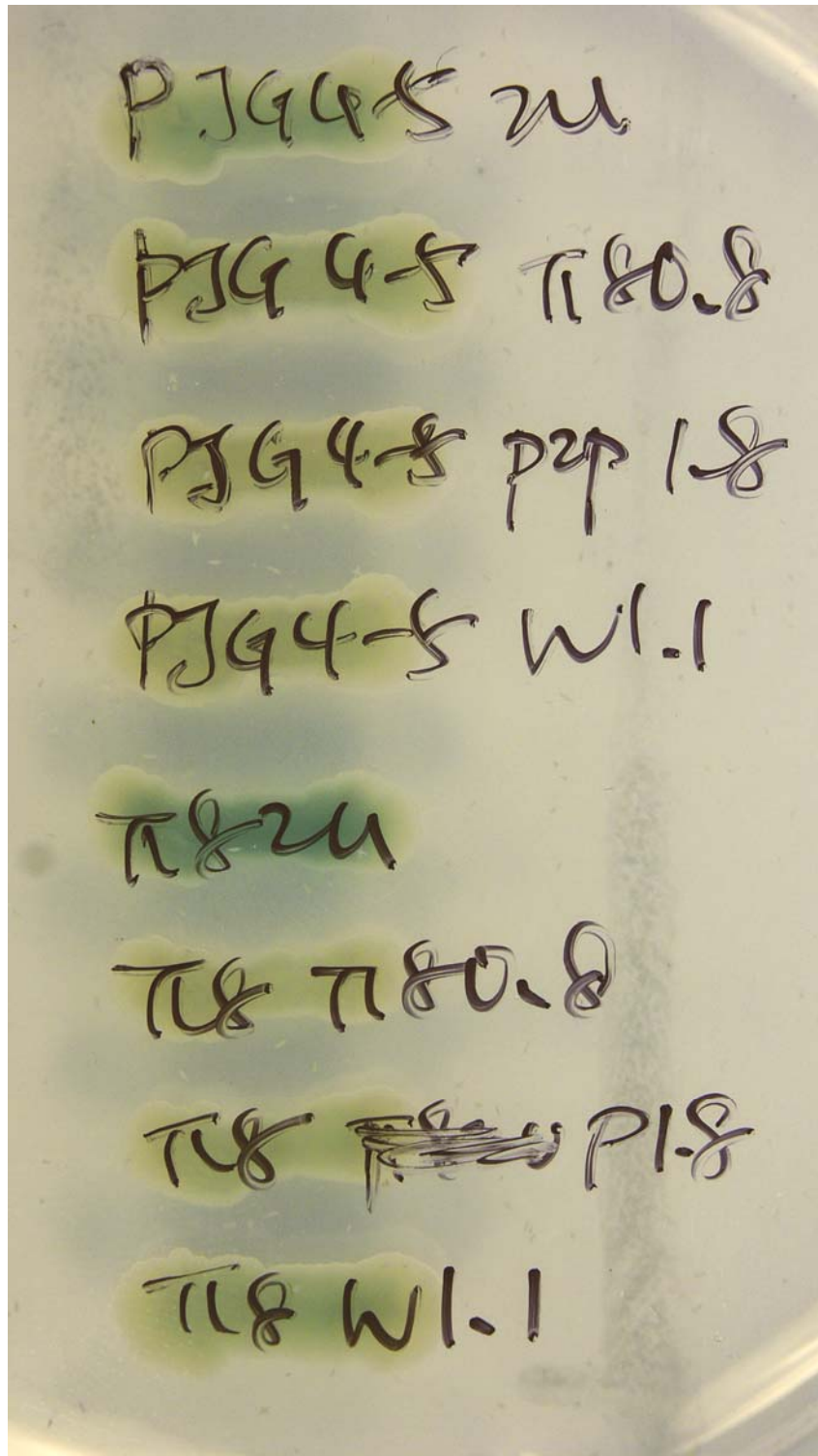


**Figure 4.10.** Yeast two hybrid analysis of protein-protein interaction in the WBM complex (A). Dilution of cell concentrations of different PAP-like transcription factors with BoTT8 (B).

*BoP2* ortholog, *MYB10*, was regulated by itself and by *bHLH3* (Espley et al. 2009), a *TT8* ortholog, suggesting that BoTT8 may be the target bHLH protein in regulating *BoP2* expression. Thus we tested the interaction between the BoTT8 and BoP2 proteins to *BoTT8*, *BoP2<sub>Pr</sub>* and *BoP2<sub>WT</sub>* promoters via a yeast one hybrid assay. However, from this analysis we did not detect any expression of the reporter gene (Figure 4.11), which suggested that the single BoTT8 or BoP2 was unable to activate the expression of *BoTT8* and *BoP2*.

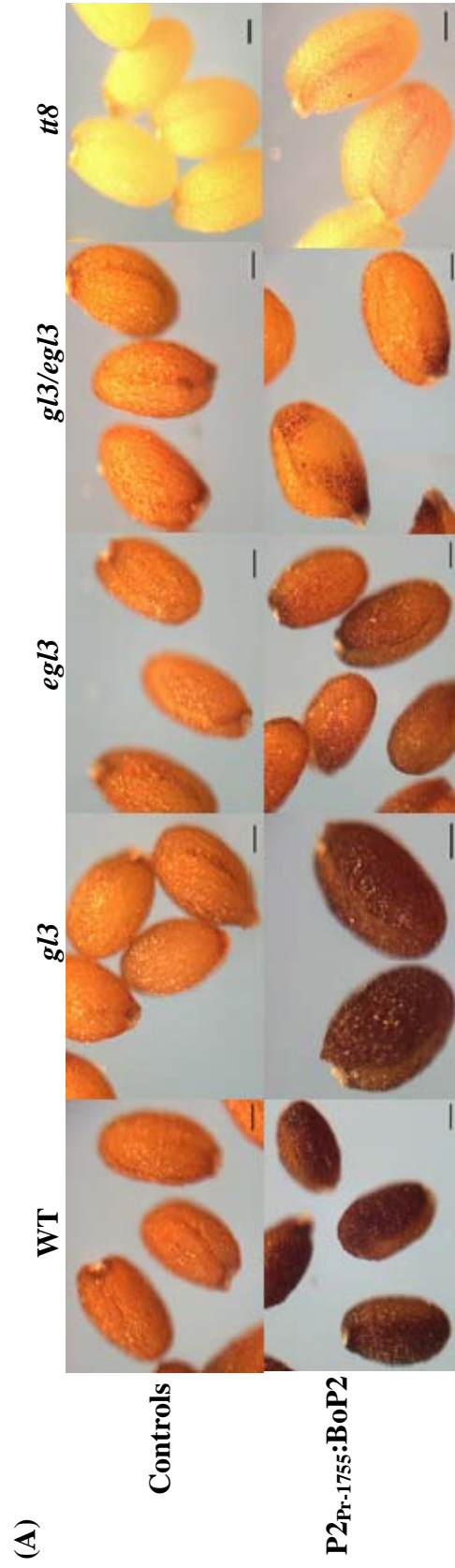
#### **4.4.6. The interaction of BoP2 with subgroup IIIb bHLH proteins in Arabidopsis**

To further ascertain the role of BoP2 in the WD40-MYB-bHLH transcriptional regulatory network, we transformed the *P2<sub>Pr-1755</sub>:BoP2<sub>Pr</sub>* construct into *Arabidopsis* *bHLH* mutant lines *tt8*, *egl3*, *gl3* and *egl3/gl3*. Reduced pigmentation was found in all mutant lines except for *gl3* when compared with *P2<sub>Pr-1755</sub>:BoP2<sub>Pr</sub>* transformants, and more anthocyanins were accumulated than in non-transformed controls (Figure 4.12). Although the *gl3* mutant line exhibited similar anthocyanin accumulation to the *P2<sub>Pr-1755</sub>:BoP2<sub>Pr</sub>* WT transformants, the *egl3/gl3* double mutant line exhibited reduced anthocyanin accumulation in seeds and young rosette leaves compared to the *egl3* mutant line. These results suggest that GL3 is involved in the BoP2 regulated anthocyanin accumulation, but with less effect than EGL3. While both EGL3 and TT8 are required in order for BoP2 to regulate anthocyanin biosynthesis, their activities could be partially complemented by each other. A previous study has shown that EGL3 and GL3 regulate *TT8* expression (Baudry et al. 2006); therefore, it still remains unclear whether the reduction in anthocyanin accumulation in *egl3* and *egl3/gl3* was the result of the interaction of EGL3 and GL3 with BoP2, or due to the reduction in *TT8* expression. Gene expression analysis of these mutant lines revealed reduced levels of *BoP2<sub>Pr</sub>* and *TT8* expression in the *egl3* and *egl3/gl3* mutant lines (Figure 4.4).



**Figure 4.11.** Example of yeast one hybrid results. Absence of coloration in the colonies indicated that BoTT8 was unable to activate the reporter gene under the control of *BoTT8* or *BoP2* promoters.





**Figure 4.12.** *Arabidopsis* transformation of *P2Pr-175s:BoP2pr* constructs in Columbia WT, and *gl3*, *egl3*, *gl3/egl3*, and *tt8* mutant lines. Seeds (A), 2-day old seedlings (B), 7-day old seedlings (C), 3-week old plants (D). Scale bars: A, 0.1 mm, B, 0.5 mm, C, 1mm.

Figure 4.12. continued

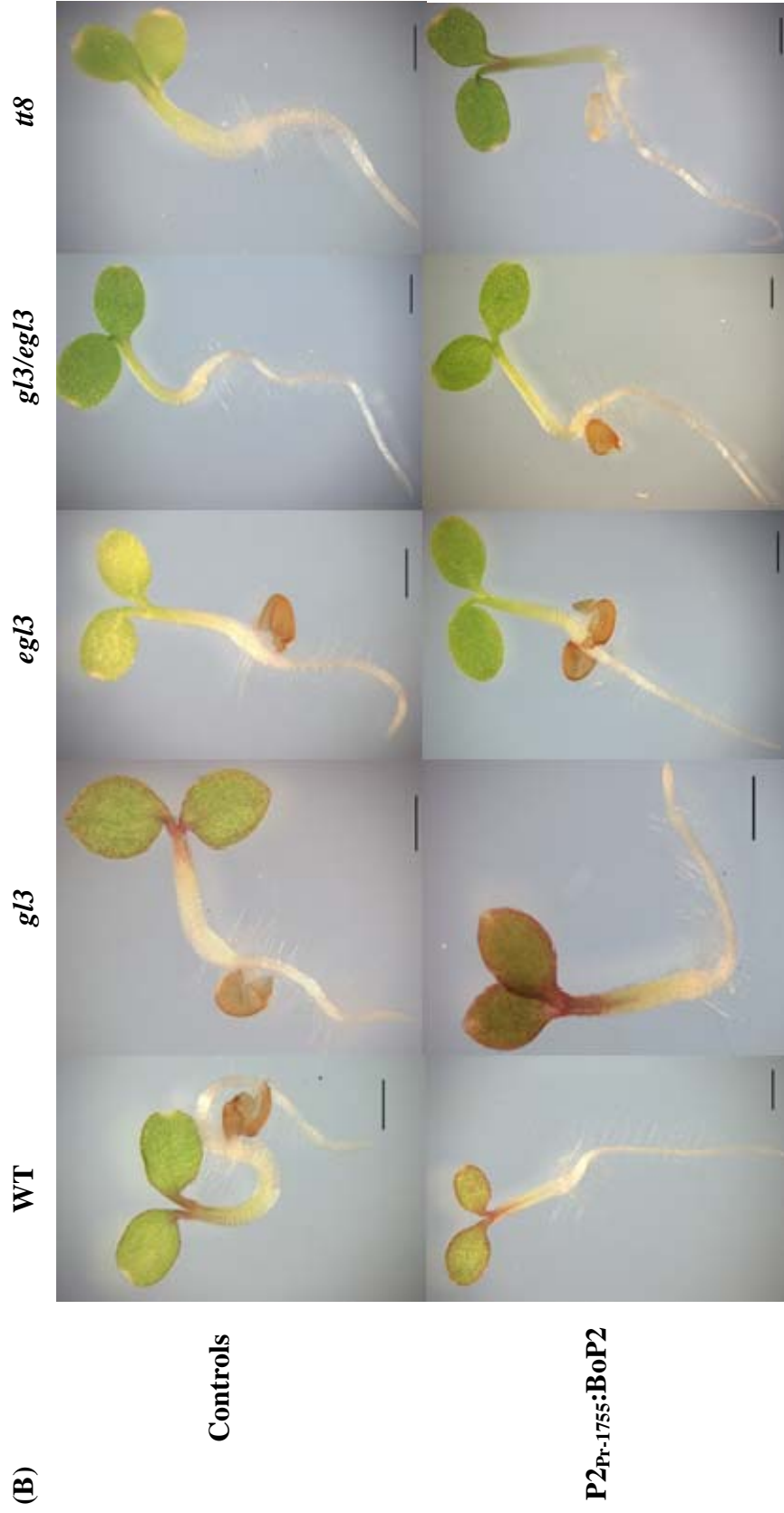
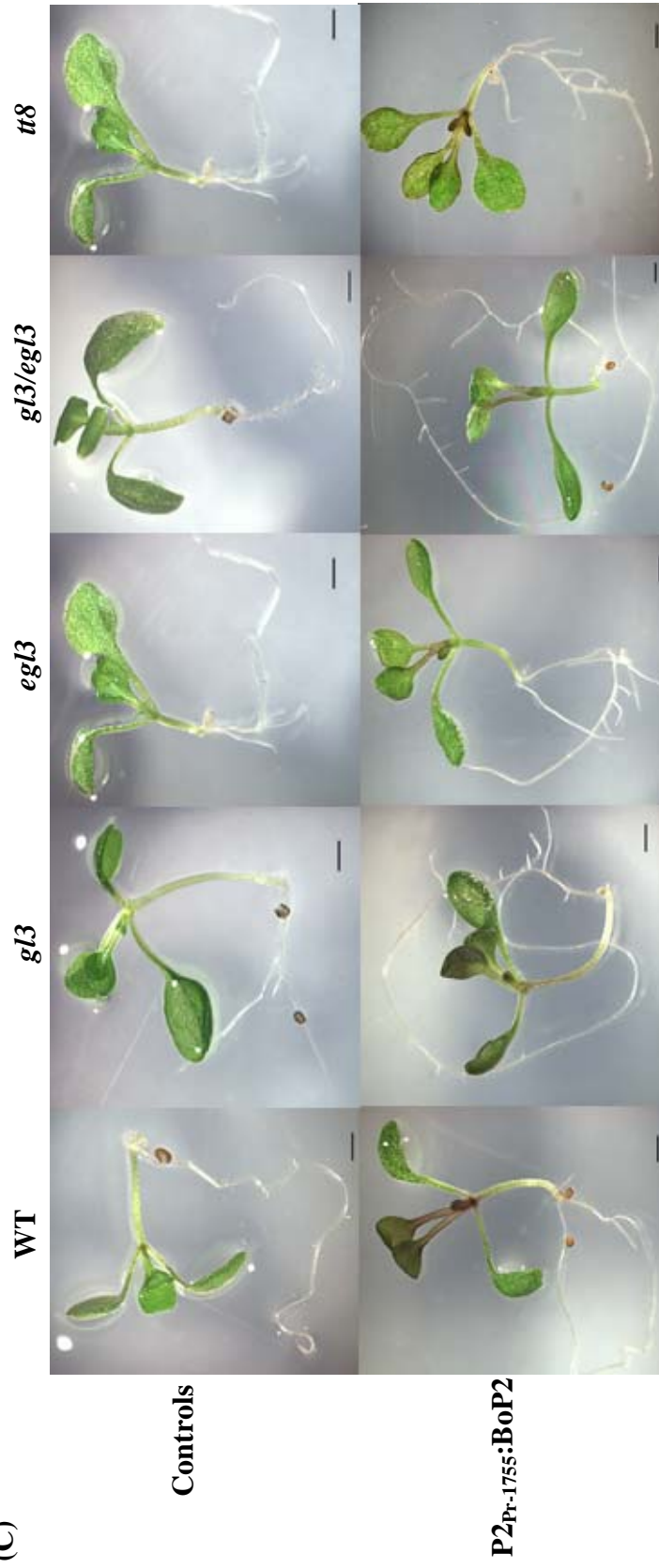


Figure 4.12. continued

(C)



(D)

Figure 4.12. continued



## 4.5. DISCUSSION

### 4.5.1. *The expression of BoTT8 and the late pathway structural genes are regulated by BoP2*

*BoP2* was found to co-segregate with the *Purple* locus within the 1898 F2 mapping population, suggesting that *BoP2* is the *Purple* gene regulating anthocyanin biosynthesis. Our phenotypic complementation results supported this hypothesis. Both *BoTT8* and *BoP2* expression was up regulated in purple cauliflower Graffiti; however, there are no sequence differences between the mutant and the wild type alleles of the *BoTT8* promoters up to position -838, suggesting that the differential expression of *BoTT8* was regulated by other genes. A previous study has shown that PAP1 in *Arabidopsis* was involved in regulating the expression of *TT8*. Thus, the expression of *BoTT8* may be regulated by *BoP2*. WD40 protein, TTG1, basic helix-loop-helix proteins, TT8, EGL3, GL3, and the MYB proteins PAP1, PAP2, MYB113 and MYB114, can form a WBM complex that is known to activate the late structural genes in the anthocyanin biosynthetic pathway (Allan et al. 2008, Gonzalez et al. 2008, Lepiniec et al. 2006, Vom Endt et al. 2002). According to our mapping data, gene expression analysis, and finding from previous studies, we hypothesized that the expression of *BoTT8* was regulated by *BoP2*; these two genes then combine to form a complex to activate the late anthocyanin biosynthetic pathway genes, resulting in the purple phenotype in Graffiti. Our gene expression analysis of the *Arabidopsis* and cauliflower *BoP2* over-expression lines and the *P2<sub>Pr-1755</sub>:BoP2<sub>Pr</sub>* lines that exhibited up-regulation of the *BoTT8* and late structural genes support this hypothesis.

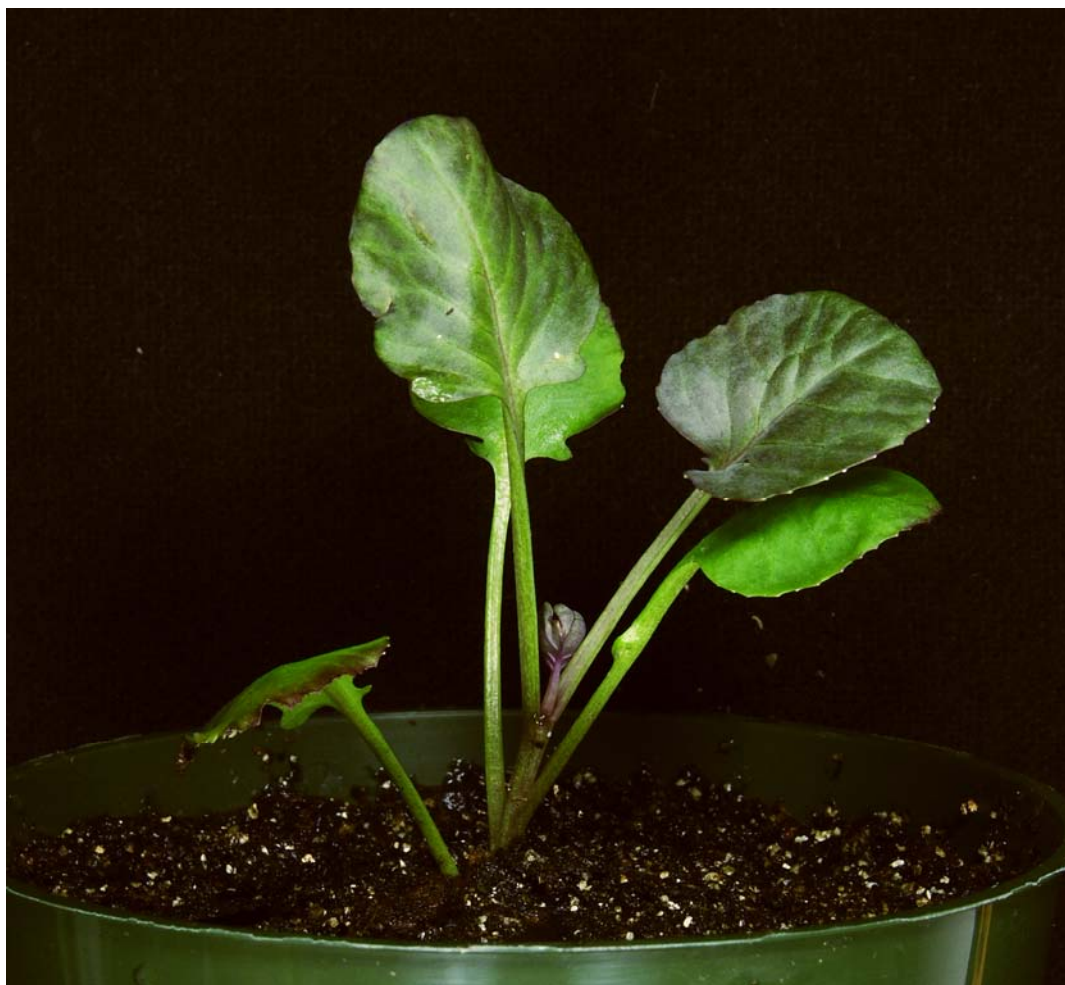
### 4.5.2. *Tissue specific anthocyanin accumulation in Graffiti*

Over-expression of *BoP2* in cauliflower and/or *Arabidopsis* resulted in anthocyanin accumulation in the endosperm (Figure 4.2.F). In *Arabidopsis*, over-expression of the

subgroup 6 R2R3-MYB genes *MYB113* and *MYB114* also resulted in a dark endosperm but not in the *PAP1* over-expressed line (Gonzalez et al. 2008). *MYB113*, *MYB114*, and *PAP2* appear in tandem in chromosome one of *Arabidopsis*. As is the case with *Arabidopsis*, the *BoP2*, *BoP3* and *BoP4* are located next to one another in cauliflower, suggesting that *BoP2* is more likely the orthologs of *MYB113* or *PAP2*. This is the first report in a dicotyledon besides *Arabidopsis*, which shows the role of subgroup 6 R2R3-MYB proteins in regulating anthocyanin accumulation in endosperm.

Graffiti also exhibits tissue-specific anthocyanin accumulation in young tissues for reasons that remain unclear. Comparison of the phenotype between the *35S:BoP2<sub>Pr</sub>* and *P2<sub>Pr-1755</sub>:BoP2<sub>Pr</sub>* cauliflower and *Arabidopsis* transgenic lines suggested that this phenotype may have arisen due to the regulation of the *BoP2* endogenous promoter. However, histochemical analysis of the *GUS* expression under the control of the *BoP2* endogenous promoter suggested that *BoP2* is expressed in the entire plant, but at a higher level in the young tissues (Figure 4.6). Moreover, a number of *35S:BoP2<sub>Pr</sub>* cauliflower transgenic lines also exhibited higher anthocyanin accumulation in young leaves than in old leaves (Figure 4.13). This suggested that other factors may also contribute to the phenotype of interest. In *Arabidopsis*, MYBL2, a repressor of the anthocyanin biosynthetic pathway, is more highly expressed in older tissues (Dubos et al. 2008, Matsui et al. 2008). Although we have yet to find an *AtMYBL2* ortholog in cauliflower, we do not exclude the possibility of an anthocyanin biosynthetic repressor existing in cauliflower. Thus the young tissue specific phenotype may be due to the combined effects of *BoP2* and the hypothetical repressor. Further screening of the anthocyanin biosynthetic repressors in cauliflower will be needed to confirm this hypothesis.





**Figure 4.13.** A *35S:BoP2<sub>Pr</sub>* cauliflower transgenic line exhibited young leaves specific anthocyanin accumulation, but at a lower level in old leaves.

#### **4.5.3. PAP-like proteins function differently in regulating anthocyanin biosynthesis**

In the yeast two hybrid study, the binding activities of the PAP-like proteins with BoTT8 followed the trend  $BoP2_{Pr} > BoP4 \geq BoP1 > BoP2_{WT}$ . Our result suggested that these four proteins may exhibit different capacities in regulating anthocyanin biosynthesis. In *Arabidopsis*, different subgroup 6 proteins interact differently with the subgroup IIIf R/B-like bHLH proteins; for example, PAP1 and PAP2 exhibit better binding activity to TT8 compared to MYB113 and MYB114 (Zimmermann et al. 2004). Over-expressing these MYB transcription factors resulted in different phenotypes in *Arabidopsis* (Borevitz et al. 2000, Gonzalez et al. 2008). Interestingly, the BoP2 wild type and mutant proteins exhibited dramatically different levels of BoTT8 binding (Figure 4.10.B). By analyzing the sequences, we found two amino acid differences between the proteins from the wild type and the mutant plants (Figure 3.10). It was likely that one or both of these two changes in the amino acids resulted in these binding activity differences. Our cauliflower transformation results indicated that the *35S:BoP2<sub>WT</sub>* lines exhibited less anthocyanin accumulation compared to the *35S:BoP2<sub>Pr</sub>* lines (Figure 4.3.C), a conclusion consistent with our yeast two hybrid result. Mutagenesis of these two single nucleotide polymorphisms will help in investigating the importance of these two amino acids in the bHLH protein interaction.

Although *BoP2* is expressed much higher in the mutant plants, low levels of expression were observed in the wild type plants. On the contrary, *BoP4* is more highly expressed in wild type plants (Figure 2.8). Taking into consideration the fact that both BoP2 and BoP4 are positive regulators of the anthocyanin biosynthesis, we concluded that the expression levels of these MYB transcription factors needs to be sufficiently high to activate the pathway. A recent study suggest that the ratio of the bHLH to MYB transcription factors governs anthocyanin production in orchid flowers (Ma et al. 2008). Moreover, the expression of PAP1 needs to be sufficiently high in



order to compete with MYBL2 for TT8 binding in *Arabidopsis* (Dubos et al. 2008, Matsui et al. 2008). Unlike *Arabidopsis* (Matsui et al. 2008), BoTT8 does not form a homodimer, suggesting that the mechanism of the WBM complex in regulating the anthocyanin biosynthesis is different from the case of the *Arabidopsis*.

Taking the expression and yeast two hybrid results together, we conclude that the amounts and activities of BoP2 and BoP4 proteins in wild type cauliflower probably not high enough to activate the anthocyanin biosynthetic pathway under normal conditions. Under stress conditions, wild type cauliflower does exhibit anthocyanin accumulation in the curds or leaves (Figure 2.10), suggesting that the pathway in wild type plants is functional.

#### ***4.5.4. The expression of BoP2 and BoTT8 may be regulated by bHLH and bZIP proteins***

*BoP2* has a retrotransposon or transposon insertion in the promoter of both the wild type and mutant alleles, respectively. This transposon insertion and a few single nucleotide insertions the mutant allele results in the generation of multiple RREs located in the promoter region. Our GUS activity assay suggests a correlation between *BoP2* promoter activity and the numbers of RREs in that promoter. Two RREs were also found within the -838 promoter region of *BoTT8*. RRE, a bHLH protein recognition site, has also been found in the promoter region of the anthocyanin structural gene, *CHS*, together with ACE, a bZIP protein recognition site, and MRE, a MYB protein recognition site, in close vicinity (Hartmann et al. 1998, Hartmann et al. 2005). ACE and MRE together govern light responsiveness, and RRE and MRE together are responsible for tissue specific expression. We also found a number of ACE motifs near the RREs in the *BoP2* and *BoTT8* promoters (Figure 4.7). Although several MYB recognition sites located in the vicinity of the RREs were found in the

promoters of *BoP2* and *BoTT8*, no identical types of MRE sites in the *CHS* promoter were found in cauliflower. Our results suggest the possibility that a bHLH protein or other transcription factor is regulating the *BoP2* and *BoTT8* over-expression.

In *Arabidopsis*, the WBM transcription regulatory complex has been shown to regulate the expression of late structural genes in anthocyanin synthesis, including *F3'H*, *DFR* and *LDOX*, and also the transcription factor itself (Baudry et al. 2006, Espley et al. 2009, Gonzalez et al. 2008, Ramsay and Glover 2005, Zhang et al. 2003). The expression of *TT8* has been shown to be regulated at the transcriptional level by the WD-repeat/bHLH/ MYB complex, including TTG1 (WD-repeat), EGL3, GL3 and TT8 (bHLH), and TT2, PAP1 and PAP2 (MYB) (Baudry et al. 2006, Nesi et al. 2000, Zimmermann et al. 2004). Recently, a mini satellite-like structure was found in the promoter of *MYB10*, an activator of the anthocyanin biosynthesis in apple. Research showed that the bHLH protein and MYB10 are capable of recognizing this repeat and further enhancing the expression of MYB10 itself (Espley et al. 2009). To test whether *BoTT8* or *BoP2* is capable of activating each other or itself, we conducted a yeast one hybrid assay. Neither the single *BoTT8* nor *BoP2* was able to activate the genes (Figure 4.11). Thus the likely bHLH protein that activates *BoP2* and *BoTT8* expression remains unknown. However, previous studies implied that *Arabidopsis* requires the existence of both bHLH and MYB proteins together to activate *BAN* and *TT8* expression in a yeast one hybrid study. Thus, it is still possible that the expression of *BoP2* or *BoTT8* is regulated by itself or by the WBM complex.

The fact that the ACEs exist in the *BoP2* and *BoTT8* promoters (Figure 4.7) suggests the possibility that an unknown bZIP protein may also be involved in regulating *BoP2* expression. *Arabidopsis* over-expressing *PAP1* growing under high temperature and low light conditions exhibited a reduction of red coloration (Rowan et al. 2009), indicating the existence of additional anthocyanin regulators. A previous

study also indicated that the *TT8* activation might be regulated by a supplementary, unknown factor as well (Baudry et al. 2006). Promoter pull down assays and cDNA library screening of transcription factors are needed to confirm whether the expression of *BoP2* and *BoTT8* are regulated by unknown bHLH and bZIP proteins.

#### **4.5.5. The network of WBM transcription factors**

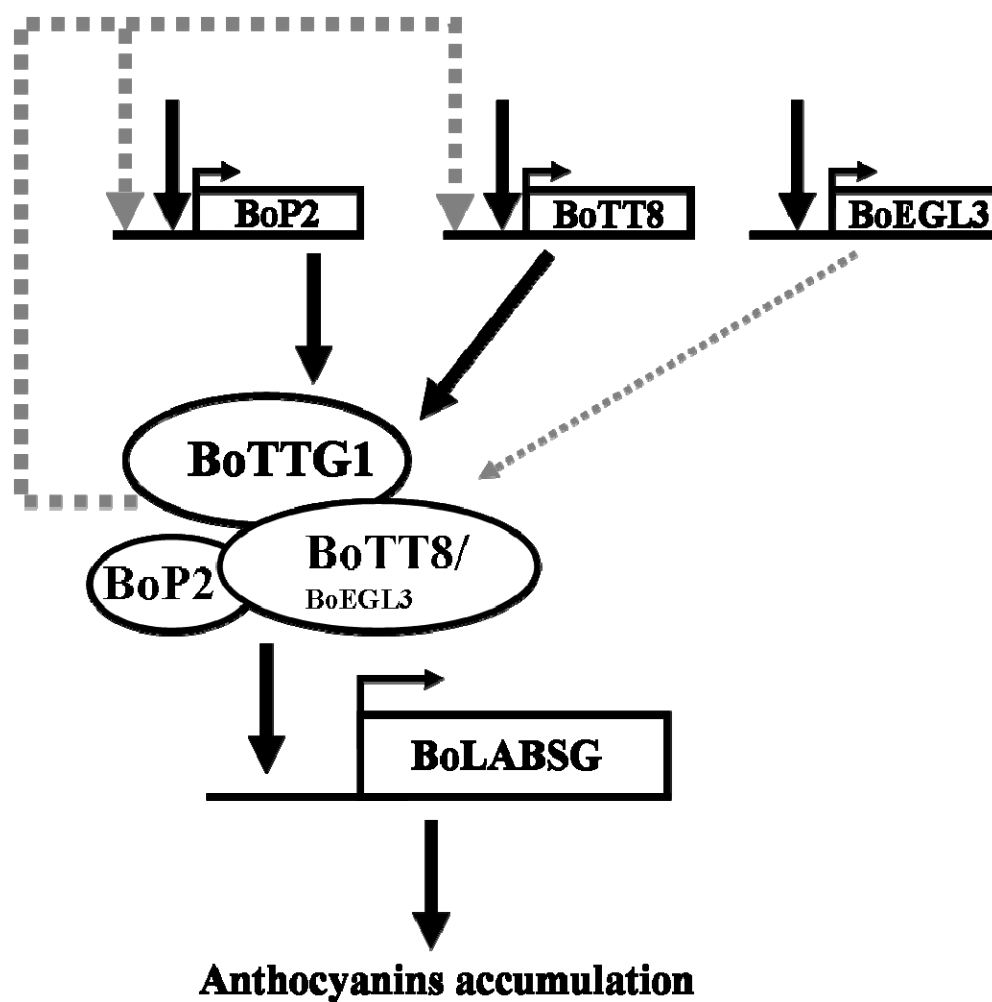
The fact that *Arabidopsis tt8*, *egl3* and *egl3/gl3* mutant lines transformed with *P2<sub>Pr-1755</sub>:BoP2<sub>Pr</sub>* constructs exhibited reduction of coloration in different tissues (Figure 4.12) suggested that TT8, GL3 and EGL3 were also involved in BoP2-regulated anthocyanin accumulation. *BoTT8* was up-regulated in *BoP2* over-expressing transgenic lines; on the contrary, the expression of *BoEGL3* seems to have no correlation with *BoP2* expression (Figure 4.4 and Figure 4.5), suggesting that *BoP2* can only regulate *TT8* but not *EGL3* expression. We did not find any *GL3* ortholog in cauliflower. Interestingly, *P2<sub>Pr-1755</sub>:BoP2<sub>Pr</sub>* transformed *Arabidopsis gl3* mutant lines exhibited high anthocyanin accumulation (Figure 4.12); however, *BoP2* transformed *gl3/egl3* mutant lines exhibited less anthocyanin accumulation than *BoP2* transformed *egl3* single mutant lines, suggesting that *gl3* may yet play some roles in BoP2 induced anthocyanin accumulation.

Previous studies in *Arabidopsis* and apple provide a number of insights into the roles of TT8, GL3 and EGL3 in regulating anthocyanin biosynthesis. TT8, GL3 and EGL3 play different roles in regulating *TT8* and *PAP*-like gene expression. TT8, EGL3 or GL3, together with the presence of R2R3-MYB proteins, PAP1 or TT2, can control TT8 expression in different tissues (Baudry et al. 2006). *MdbHLH3*, an *AtTT8* ortholog, and *MdbHLH33* can regulate the expression of *MdMYB10*, a *PAP*-like gene in apple (Espley et al. 2007, Espley et al. 2009). *EGL3* plays a more prominent role in regulating *TT8* expression than *GL3* (Gonzalez et al. 2008). EGL3 and GL3 can

additively participate in *TT8* regulation (Baudry et al. 2006, Gonzalez et al. 2008). *TT8* is the direct target of GL3, while *PAP2* is the secondary target of GL3, and *PAP1* is not a GL3 target (Gonzalez et al. 2008). Also, TT8, GL3 and EGL3 play different roles in regulating anthocyanin biosynthetic genes. PAP1/PAP2 is able to bind with TT8/EGL3/GL3 as a complex to regulate *DFR* expression (Zimmermann et al. 2004). *F3'H*, *DFR*, *LDOX* are direct targets of GL3 (Gonzalez et al. 2008). Over-expression of *GL3* results in no effect to *DFR* and *LDOX* expression while wild type *EGL3* does have a significant effect on *F3'H* expression (Gonzalez et al. 2008).

In our study, we found that RREs in the promoter may play an important role in *BoP2<sub>Pr</sub>* over-expression (Figure 4.7 and Figure 4.8). BoTT8 could bind with BoP2 to form a complex (Figure 4.10). Over-expression of *BoP2* resulted in increased expression of *BoTT8* and lower pathway structural genes, but did not effect on *BoEGL3* expression (Figure 4.4 and Figure 4.5). The expression levels of *AtTT8* in *P2<sub>Pr-1755</sub>:BoP2<sub>Pr</sub>* transformed *Arabidopsis* lines were as follows: *gl3*  $\geq$  WT  $>>$  *tt8*  $>$  *gl3/egl3*  $>$  *egl3* (Figure 4.4). The expression levels of BoP2 in the *P2<sub>Pr-1755</sub>:BoP2<sub>Pr</sub>* transformed *Arabidopsis* lines were as follows: *gl3*  $\geq$  WT = *tt8*  $>$  *gl3/egl3*  $>$  *egl3* (Figure 4.4).

Taken together, the functional differences between GL3 and EGL3 in regulating the expression of transcription factors and late structural genes accounted for the phenotypic differences between the *gl3*, *egl3* and *gl3/egl3* mutant lines transformed with *BoP2*. The WBM complex involved in regulating the expression of transcription factors and late structural genes explained the up regulation of *BoTT8* and the lower pathway structural genes while *BoP2* was up regulated. The presence of several RREs in the *BoP2<sub>Pr</sub>* promoter, the data on the *Arabidopsis* transgenic lines, and the *BoTT8* orthologs involved in regulating the expression of *MYB* genes suggests that BoTT8 is the main bHLH protein involved in BoP2 induced anthocyanin



**Figure 4.14.** The possible model for the BoP2 induced anthocyanin accumulation in purple cauliflower. The expression of *BoP2* and *BoTT8* may be the result of autoregulation. The dotted line indicates the need for further experimental verification.

accumulation while GL3 and EGL3 may also have a number of effects. Thus, we propose a possible model here for the BoP2 induced anthocyanin accumulation in purple cauliflower (Figure 4.14). The expression of *BoP2* and *BoTT8* may be controlled by an auto-regulatory mechanism. BoP2, BoTT8/BoEGL3, and BoTTG1 can form a complex to regulate the expression of late anthocyanin biosynthesis structural genes and transcription factors, while EGL3 appears to have a minor involvement in this complex. More studies, such as using the glucocorticoid receptor (Baudry et al. 2006, Lloyd et al. 1994) fused with BoP2 and EGL3 to detect their primary or secondary targets; over-expression of *BoP2* via the 35S promoter in different *bHLH* and *TTG1* mutant lines to examine the relationship between these three bHLH transcription factors; and yeast two hybrid between BoEGL3 and BoP2 are needed to verify this model.

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## CHAPTER 5

### SUMMARY AND FUTURE DIRECTIONS

#### 5.1. SUMMARY

In this study, we characterized an interesting purple cauliflower cultivar Graffiti which exhibited high level of tissue specific anthocyanin accumulation in young siliques, young leaves, young flower buds, curds, and seed endosperm (Figure 2.2). Cyanidin 3-(coumaryl-caffeyl) glucoside-5-(malonyl) glucoside was found to be the predominant anthocyanin accumulated in the purple curd tissue (Figure 2.6). The *BoF3'H*, *BoDFR*, *BoLDOX*, *BoTT8*, *BoP2*, and *BoP4* genes exhibited differential expression patterns between the wild type and mutant plants (Figure 2.7 and Figure 2.8). We have identified *BoP2* to be the *Purple* gene controlling the ectopic anthocyanin accumulation, and found that over-expression of *BoP2* resulted in *BoTT8* up-regulation (Figure 4.4 and Figure 4.5). These two proteins, together with BoTTG1, appear to form a WBM transcription factor complex (Figure 4.10) which up-regulates the expression of the late pathway anthocyanin biosynthetic genes, including *BoF3'H*, *BoDFR* and *BoLDOX*. An autonomous DNA transposon insertion at the -373 position of the *BoP2<sub>Pr</sub>* promoter generated several RREs within this promoter (Figure 4.7), thereby leading to the ectopic expression of the *BoP2* mutant allele. GUS activity assays suggest that promoter activity is correlated with the numbers of RREs in the promoter region (Figure 4.8). The RRE motif is a bHLH protein recognition site, indicating that bHLH proteins are likely to be involved in regulating *BoP2* expression. According to a previous study (Espley et al. 2009), BoTT8 is conceivably the major bHLH protein controlling *BoP2* over-expression. Over-expression of *BoP2* resulted in the up-regulation of *BoTT8* but not of *BoEGL3* (Figure 4.4 and Figure 4.5); therefore, BoTT8 is plausibly the major transcription factor responsible for *BoP2* expression.

Taken together, we put forward a possible model for the BoP2 induced regulation of anthocyanin production in purple cauliflower (Figure 4.14). The expression of *BoP2* and *BoTT8* may be controlled by an auto-regulatory mechanism, and further experiments will be needed to confirm this model.

## **5.2. FUTURE DIRECTIONS**

### ***5.2.1. The numbers of RREs correspond with the BoP2 expression level***

Several RREs, also known as the E-Box (Hartmann et al. 2005), are located in the BoP2 promoter region (Figure 4.7). GUS activity assays revealed that the RREs may play an important role in regulating *BoP2* expression. From previous studies on apple, E-Boxes and G-Boxes located in the MYB10 promoter have been found to be correlated with MYB10 over-expression (Espley et al. 2009). To confirm this hypothesis, activity assays of BoP2 promoters with different RRE deletions should be conducted.

### ***5.2.2. Isolation of the proteins that control BoP2 and BoTT8 expression***

According to our promoter sequence analysis and GUS activity assay results, bHLH proteins may play an important role in regulating *BoP2* over-expression. Sequence analysis of the *BoTT8* promoter revealed the presence of RREs, suggesting that the expression of *BoTT8* may also be regulated by the bHLH proteins. Previous studies indicated that the subgroup IIIf bHLH proteins, including TT8, EGL3 and GL3, are likely to be the most plausible candidates (Baudry et al. 2006, Espley et al. 2009). To further investigate whether any of these subgroup IIIf bHLH proteins is the *BoP2* regulator, electrophoretic mobility shift assays (EMSA), or yeast one hybrid assays with co-expression of BoP2 is needed to confirm the binding activity of subgroup IIIf bHLH proteins to the *BoP2* promoter. A previous study has described how the

expression of *TT8* in *Arabidopsis* is regulated by the subgroup IIIf bHLH and subgroup 6 MYB proteins (Baudry et al. 2006). RREs were also found in the *BoTT8* promoter, suggesting that the subgroup IIIf bHLH proteins may regulate the expression of *BoTT8* as well. Thus, yeast one hybrid assays or EMSA will be needed to confirm this hypothesis.

In Chapter four, we also described how the bZIP proteins may be involved in regulating the expression of *BoP2* and *BoTT8*. The bZIP and MYB recognition sites, ACE and MRE, located in the CHS promoter, are responsible for light related gene expression regulation. To determine which of the bZIP proteins is involved in regulating *BoP2* and *BoTT8* expression, yeast one hybrid protein library screening using the *BoP2* or *BoTT8* promoter may help in isolating the candidate proteins.

### **5.2.3. The regulatory network of MYB, bHLH and WD40 proteins**

A previous study in *Arabidopsis* has shown that *TT8* is the direct target of GL3 and TTG1, *PAP2* is the secondary target of GL3, and *PAP1* is not a GL3 target (Baudry et al. 2006, Gonzalez et al. 2008). The structural genes *DFR* and *LDOX* are the direct targets of GL3 and TTG1; however, *F3'H* is the secondary target of TTG1 but the direct target of GL3 (Gonzalez et al. 2008). According to these data, the regulatory network linking MYB, bHLH and WD40 proteins to the anthocyanin biosynthetic genes and transcription factors appears to be quite complex. To obtain a more complete picture of the entire regulatory network, transgenic *Arabidopsis* with transcription factors fused with glucocorticoid receptor will allow us to identify the direct or secondary targets of the proteins (Lloyd et al. 1994). Subgroup 6 R2R3-MYB, *BoP1*, *BoP2* *BoP3* and *BoP4*, or subgroup IIIf bHLH proteins, *BoTT8*, *BoEGL3* and *BoGL3*, under the control of CaMV 35S promoter, can be fused to the glucocorticoid receptor and transferred to the corresponding transcription factors mutant lines in

*Arabidopsis* and treated with dexamethasone (DEX) and cycloheximid (CHX). Gene expression analysis of these lines will allow us to identify the direct or the secondary targets of the mutant proteins.

#### ***5.2.4. Potential anthocyanin biosynthetic repressor in purple cauliflower.***

Graffiti exhibits tissue-specific anthocyanin accumulation in young tissues. The reason for this interesting phenotype remains unclear. Recently, MYBL2, a repressor of the anthocyanin biosynthetic pathway, was found in *Arabidopsis*, where it is more highly expressed higher in the old tissues (Dubos et al. 2008, Matsui et al. 2008). The mechanism by which MYBL2 regulates the anthocyanin biosynthesis is through competing for bHLH protein binding with the subgroup 6 R2R3-MYB proteins in the WBM complex, thereby preventing the binding of this complex to the promoter of the structural genes to repress the pathway. In our study, we put forward the possibility of WMB complex involvement in regulating the *BoP2* and *BoTT8* expression. Therefore, a repressor like MYBL2 may be one of the factors contributing to the interesting phenotype by prohibiting the WMB complex from binding to the promoters of *BoP2* or *BoTT8*. Although we have yet to find an *AtMYBL2* ortholog in cauliflower, we did not exclude the possibility that an anthocyanin biosynthetic repressor exists in cauliflower. A number of 35S:*BoP2*<sub>Pr</sub> transgenic plants also exhibited higher anthocyanin accumulation in the young leaves, hinting the existence of other factors than the promoter activity. Further screening of the anthocyanin biosynthetic repressors in cauliflower will be required to confirm this hypothesis.

#### ***5.2.5. The amino acid difference between BoP2<sub>Pr</sub> and BoP2<sub>WT</sub> influences their binding activity to BoTT8***

BoP2<sub>Pr</sub> and BoP2<sub>WT</sub> have only two amino acid differences at the 14 and 159 positions (Figure 3.10). Our yeast two hybrid assay revealed however, that there existed dramatic difference between their binding activities to BoTT8 (Figure 4.10.B). Our results suggested that one or both of these amino acids may play an important role in the protein binding activity. A previous study revealed the location of the critical motif within the 53 to 114 region. Therefore, we were unable to determine which of these two amino acids is responsible for contributing to the protein binding activity. Point mutagenesis to restore the amino acids from BoP2<sub>WT</sub> to those in BoP2<sub>Pr</sub> will help us identify the roles of these two amino acids.

### **5.3. CONCLUSION**

In conclusion, we have identified a R2R3-MYB transcription factor, BoP2, responsible for the ectopic anthocyanin accumulation in the purple cauliflower, Graffiti. Single nucleotide polymorphism and autonomous DNA transposon insertion in the promoter region of *BoP2* led to its over-expression. This is the first case in vegetables where a R2R3-MYB protein results in seed endosperm and young tissue specific anthocyanin accumulation. Further experiments as described above may help us create a more complete picture on the regulation of anthocyanin biosynthesis in vegetables, and shed more light on a new strategy for nutritional improvement of food crops.

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## APPENDIX 1

### PRIMERS DESIGN

Gene specific primers were designed based on the sequences from both the *Arabidopsis* (TAIR, [www.arabidopsis.org](http://www.arabidopsis.org)) and *Brassica Oleracea* (TIGR, [www.tigr.org/tdb/e2k1/bog1](http://www.tigr.org/tdb/e2k1/bog1)) databases. To design primers for cauliflower genes, *Arabidopsis* gene sequences were obtained from the TAIR database and blasted to the TIGR database to acquire the sequences of the orthologs. The primers design was based on the sequence information of the *Brassica Oleracea* orthologs. Cauliflower orthologs were then amplified and cloned to obtain the sequence information for further primers design. The adjacent gene sequences were obtained using the genome walking method.

**Table A1.1.** List of primers used in this study

Primer number	Gene	Sequence (5' to 3')
1	CHS	GAAGTCCCTAAGCTAGGCAAAGAGGC
2	CHS	GGTCAGAACCCAAACAAGACACCCC
3	CHI	ACCGTCCGTCATCTCACCGGC
4	CHI	CAGGAATGCTATCGTCTTTTCGAAAACGC
5	F3H	GGAGAGATCTGTCGACAGATCGTTGAG
6	F3H	TAATCTGCTCGAGTTGGAGTTCACCAC
7	F3'H	GCCCATCATCGGAAACCTCCCTC
8	F3'H	AGCTTCTCCGGCGTAACTCCTCC
9	DFR	CTTCTTGATTTGCCAAACGCGAAGAC
10	DFR	GGGAGAAAACCCTTTTGACGACATGTC
11	LDOX	ATGGTGGCAGTTGAAAGAGTTGAGAG
12	LDOX	GAAACTTAGCCGAGACTCAACACTCA
13	UGT	ATGGCCGAACCAAACCACAAGCTTC
14	UGT	CCATTCAGGTTTCGAGCTCCGTACATG
15	UGT	CTTGTTCTCCGATGGATTTCGACGA
16	UGT	TCCTCGTCTGATGATCTGAAAAGATCGG
17	Actin	CCGAGAGAGGTTACATGTTTACCAC
18	Actin	GCTGTGATCTCTTTGCTCATACGGTC
19	TTG1	CAACAACAGCAAGACGAGCGAGTT



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20	TTG1	GACCACTGCAACTGGTTAATCTCCG
21	TTG2	GGCACACATCGAGCAGGTCCTAA
22	TTG2	GCTTCTGCTTGGATCATCAAGCTCTC
23	TT8	CAATCTGTGGGGTGGACTTATAGTGTCT
24	TT8	AGATCATCATGTCCATGTGTGTATCTA
25	TT2	GAGTTAAACAGAGGAGCTTGGACCGATC
26	TT2	AACCATCAAAGTCCATCAAATCAGAGAAG
27	FUS3	TCATGGGTTATCGGCGTCTGTGC
28	FUS3	TGAAGGTCCAAACGTGAAGACCATCCA
29	RNS1	GGCCAGGATCATACTGTGACACACAG
30	RNS1	TCGATAACCGATTCCGAGCAAGTACC
31	ANL2	GCAATGAGGAATCCAATGTGCAACAAC
32	ANL2	CGAATCTTCTGGACGGTGCATGATATT
33	EGL3	TGGGGAGATGGATGCTACAATGGAGA
34	EGL3	CGAGTGAAGACTTTGCTATCGGCGG
35	P1	CGGCTGAAGAAGACAGTCTCTTGAGGCG
36	P1	CGTACCTTTTGGAACGAGCCCGTCA
37	P2	TCTAGCAAACCTTTCCCACCACCTATTCTGA
38	P2	TGAAGCCAAGTATCAAGAGAGGAAAACCTCAA
39	P3	GTCCAAACGGTTGAGAAAAGGTGCATAT
40	P3	TCCAAAGTTGCTCAACGTCAAACGC
41	P4	CCAAACGGTTGAGAAAAGGTGCATGG
42	P4	AGCGTCGTTGAATCCAAGGCATAGG
43	TT8	TCCACCTTCCTCCATTAGATTTCATCATGTCCA
44	TT8	ACGGTGCAATAAAGACGAGGAAGACAAC
45	EGL3	TGAACATCGTCGTCCATATTTACATGGTTACTC
46	EGL3	TGAATCCTCAGCTTCCGGTAGCTCTCAG
47	GL3	TCTTCTTCTTCAGGCGTTGCTGCCG
48	GL3	ACGTCGTCGTTTCTTGATCAAATGACAA
49	PAP1	TCTGAGTAAGAAACATGAACCGTGTGTAAGATA
50	PAP1	TGTTGTCGTCGCTTCAGGAACCAAAAAT
51	PAP2	TGAAGCCAAGTATCAAGAGAGGAAGACTTAGCA A
52	PAP2	AAACGCCAAAGTGGCCCCATGTTCA
53	MYB113	TGAAGCCAAGTATTAAGAGAGGAAAACCTCT
54	MYB113	ACCCAACACATCTGCCTCCAGTAAA
55	MYB114	TGAAGAAGATAGTCTCTTGAGGCAGTGATTGG
56	MYB114	TTTTGGGCCGGTGTATTAGGAGGGGTTA
57	P2	GCTTGAGGAAAAGGGTTTATATTGTGGCT
58	P2	GTCGGAAGTGTTTAAAGAGAGAACATGAT
59	P2	TTAGAGCTATCGGGGCGTGTCTAGAGA
60	P3	GGTGCATGAAGCCGTTCTCTCTTTTAATC
61	P3	TGAAACTGAAGGAGTACTCCCTTGGTAAAC

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62	P4	CCCAAGTGTATCAGCTACAGAAGAAGCCT
63	P4	TCGTTGTTGGTTTGTCTGTTTCAAGTGAAAT
64	P4	AAGGAAGGTTTCTCTTTTTCATTTGGGTCTTAC
65	P2 <sub>Pr-1755</sub> :P2 <sub>Pr</sub>	AACGAGCTCTAAAACGATTGTTGGCTTACCC
66	P2 <sub>W-1061</sub> :P2 <sub>WT</sub>	TGCAGAGCTCATTATGATATCGCGACCAATTAGA
67	P2	TGGAGTCGACTCAAGTTCCAGTTTCTCCATCCA
68	P2	TCGGAGCTCCATGGAGGGTATGTCCAAAG
69	P2 <sub>pro</sub>	ACCCTCCATGGACCAGCGATATATAATATAAGT
70	P2 <sub>Pr-373</sub> /P2 <sub>WT-378</sub>	TGCGGCCGCAATCATTAAATAGTACTTTCT
71	P2 <sub>Pr-1079</sub>	AAGCGGCCGCATATGGTGATTGAAAAGAAAAGA
72	P2 <sub>Pr-1755</sub>	GAAGCGGCCGCCTCTAAAACGATTGTTGGCTTA
73	P2 <sub>WT-1061</sub>	ATCGCGGCCGCTTAGAATCTCCCTCCATCCA
74	Y2HTTG1	ACAATGTCGACCTCAAACCTCTGAGGAGCTG
75	Y2HTTG1	GAGAGAGAATTCATGGACAACCTCAGCTCCA
76	Y2HTT8	AGGTTAGAATGTCGACACTAGAGTTTATTATT
77	Y2HTT8	TCTCGGGAATTCATGGATGAATTAAGTATT
78	Y2HP1	AGTGGGTCGACACTAATCAAGTTCTACAGT
79	Y2HP1	AATCTTGAATTCATGGAGGATTCGTCCAAAG
80	Y2HP2	AGTGGGTCGACATCAAGTTCCAGTTTC
81	Y2HP2	TATCGCGAATTCATGGAGGGTATGTCCAAAG
82	Y2HP3	AGTGGGTCGACATCAAGTTCTCGTCTC
83	Y2HP3,4	TATAGCGAATTCATGGAGGGTTCGTCCAAAC
84	Y2HP4	AGTGGGTCGACATCAAGTTCTCGCCTC
85	EGL3 map	TCAAGCAATATCCCTTCTCTCCAAGCAC
86	EGL3 map	AGAGTCAAAGGTTAGGTCAGATTCAAGAACAG
87	EGL3 map	GGCGTGGTTCGCGTTTTTCATCTCCAACCTTATT
88	EGL3 map	CACCAAAAGGACACACCCGAAGATAGCGGTT
89	TT2 map	AAACTGCTTGCCTGACTTGTGTTAT
90	TT2 map	TTCAGAGAGGAAGAAGTCGAAGATCTC
91	TT2 map	CTTAGAAAATATTACCCTGTACGACTCAGA
92	TT2 map	CCAGAACATGATCTTTCTAGCTTCGG
93	Y1HTT8	TCGAGCTCTATTGGGTTTTATAAGACATTAACAAGGGA
94	Y1HTT8	ATTCAGTAGTCTTTCCCCGAGATGAAAACCTGTGA
95	Y1HP2	ACCACTAGTGGACCAGCGATATATAATATAAGTATCT
96	Y1HP2P	AACGAGCTCTAAAACGATTGTTGGCTTACCC
97	Y1HP2W	TGCAGAGCTCATTATGATATCGCGACCAATTAGA
98	Y1HTT8	AGCTCGAGCTAGAGTTTATTATTATATATGATTGA
99	Y1HTT8	AGCAGAGAATTCATGGATGAATTAAGTATT
100	Y1HP2	AGCTCGAGTCAAGTTCCAGTTTCTCC
101	Y1HP2	AGCAGAGAATTCATGGAGGGTATGTCCAAAG

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